

Post-Kyoto agendas

The Kyoto meeting on climate change was a small triumph for the international community and a benchmark in the process of consensus. But much bigger challenges lie ahead, for scientists and politicians alike.

Who emerges with most credit from the Kyoto summit? The United States has clearly gone further to achieve a deal — agreeing to a 7 per cent cut in its emissions without quite securing the involvement of the developing countries in the project — than its detractors had envisaged. President Bill Clinton and Vice-President Al Gore would have taken much of the blame if the meeting had collapsed for not standing up more firmly to the fossil-fuel and automobile lobbies, and can take credit for defying the pessimists and allowing it to succeed.

The conference chairman, Raul Estrada Ouyela of Argentina, deserves congratulation for his witty and successful brow-beating of delegates. Australia, India, China and Saudi Arabia deserve credit for not derailing the talks. Bouquets, too, to the Japanese hosts for picking the UK deputy prime minister, John Prescott, for a diplomatic role which he amply fulfilled, and for the fact that the agreement that emerged is close to Japan's compromise proposal.

Now for the political reverberations. A tentative agreement has been reached between the developed countries, but more work — and some flexibility from the developing countries — is necessary before the next meeting of the parties in Buenos Aires next year if a full treaty is to be ratified.

As the Clinton administration attempts to prepare the ground for ratification by the US Senate, the onus will fall on scientists to convince both Senate and the wider public that scientific knowledge of climate change is sufficiently robust for the economic upheaval required to implement the targets agreed at Kyoto. Given the timetable of Clinton's implementation plan, researchers have at most five years left to do what they have not quite achieved in the past ten, and convince a wider audience in the United States that the science of global climate change represents such a foundation. Most

senators — like most of the people they represent — have yet to pay enough attention to form a well-founded view on the matter.

Over the next few years, some of the toughest scientific challenges will be in the areas of emissions measurements, verification and regional impacts. A new report issued last week by the Intergovernmental Panel on Climate Change, *The Regional Impacts of Climate Change*, does a useful job in spelling out regional, ecological and hydrological sensitivities to global warming. But it is also clear that several years, at least, of development of models and computing will be required before regional predictions can inspire confidence.

It is salutary to realize that, all else being equal, whatever was agreed at Kyoto could make only a small and undetectable fraction of a degree difference in the average temperature in 2100. That makes Kyoto's achievements important more as a concentrator of minds. And Kyoto suggests that a top-down approach to emissions reduction, even with more determination on all sides, will never be sufficiently effective to yield the scale of reductions that models suggest is required. But it is questionable whether market forces will also contribute much, even if energy prices were to rise. Even where there is a clear and immediate threat, such as malaria, the combined forces of politics, education and business have proved thoroughly inadequate in alleviating the problem.

If the developed countries are serious about meeting the targets they have set for themselves — and readers should note that the summit delivered no penalties for overshooting — they will encourage very considerable additional public and private sector investments in the research and development of new energy sources and energy conservation techniques. One of Kyoto's achievements is to have highlighted the need to go much further in that direction than is at present envisaged. □

World wide wastrels?

The Internet has aggravated a problem with youthful interest, but also provides a solution.

You know that feeling of resentment. Some kid somewhere — or worse, some undergraduate — writes you a letter asking vaguely for help with a science project related, more or less, to your field of research. What to do? Curse and ignore? Mutter "don't they teach them to use libraries any more?" and reply with a peremptory instruction not to be so lazy? Nowadays, of course, letters have been superseded by e-mails. Thanks to the Internet and the World Wide Web, such 'cold calling' by total strangers is all the more frequent. And it's not as if they are your own offspring, to whom you might respond in the patient, considerate and generous manner that reflects the real you.

Malcolm Buck, a geologist at the University of New South Wales (m.buck@unsw.edu.au), was sufficiently provoked by one polite but all-encompassing request about volcanoes ("If you could please e-mail me with any information on the [nine] topics listed...") to undertake a survey of how other Earth scientists are dealing with this growing burden.

Almost all of the 74 respondents — from nine countries — agreed that a reply of some kind is necessary, but rightly concluded that one should avoid doing the student's work for him or her. A generalized reply, offering encouragement and recommending useful sources of information (including the local reference librarian), seems to be best. But recommending books is problematic, as many school and town libraries have inadequate collections.

Fortunately, help is at hand in the shape of the very monster that exacerbated the problem — the World Wide Web. Volcanology is blessed with many excellent web sites, including some with near-real-time information on erupting volcanoes. Other disciplines may be less cyberspatially well endowed, but with minimal effort — often simply a form response — scientists can direct students to sources of information that can turn a casual enquiry into an adventure. Better that than to miss an opportunity to foster scientifically minded enthusiasm. □



Kyoto agreement creates new agenda for climate research

[KYOTO] Last week's adoption of the 'Kyoto protocol' on climate change has been welcomed by climate scientists as "a step in the right direction" as they prepare to respond to the challenges arising from the conference.

Having already played an important role in the run-up to the protocol's adoption, the scientific advisory bodies to the conference have been asked to provide solutions to two immediate challenges.

First, scientists need to lay out in detail how greenhouse gas emissions are to be measured and verified — including the release and absorption of carbon from changes in land use and forest management. Second, scientists have been asked to come up with a mechanism for 'trading' emissions before the next climate conference in Buenos Aires in a year's time.

After ratification by at least 55 countries, the protocol will require developed countries to reduce emissions of a combined 'basket' of six gases by an average of 5.2 per cent from their 1990 levels between 2008 and 2012. Gases will be calculated from both their sources and 'sinks'. There are no commitments — voluntary or otherwise — for developing countries.

Most of the 39 developed countries covered by the protocol will reduce emissions by 8 per cent. The United States will have to



reduce emissions by 7 per cent, and Japan by 6 per cent. Russia, New Zealand and the Ukraine will be allowed to stabilize emissions at 1990 levels. Australia will be allowed to raise its emissions by 8 per cent above 1990 levels.

Reductions to three gases — carbon dioxide, methane and nitrous oxide — will be made relative to 1990 levels. Countries can choose whether to use 1990 levels or a 1995 baseline when reducing emissions of the other three gases: hydrofluorocarbons, perfluorocarbons, and sulphur hexafluoride.

Reducing a combination of gases poses governments with a difficulty, as the relative

impact of the different gases is not the same: one tonne of carbon dioxide does not affect climate in the same way as one tonne of methane, so the quantities of each country's emissions of each gas cannot be added in a linear fashion.

One of the most controversial parts of the protocol allows higher-emitting developed countries to achieve their reduction targets by buying emissions 'credits' from lower polluting developed countries under a scheme known as emissions trading (see Briefing, *Nature* 390, 215; 1997).

A similar scheme — known as joint implementation — will allow higher polluting countries to obtain emissions credits by building 'clean' technology projects in lower polluting developed countries. The United States will achieve some of its target by buying credits from Russia and the Ukraine, whose emissions are already below 1990 levels.

Competing schemes for emissions trading have already been developed by the World Bank (see *Nature* 390, 7; 1997) and the United Nations Conference on Trade and Development. Frank Joshua, head of greenhouse emissions trading at UNCTAD, believes that the details of a practical trading mechanism will not take long to work out.

In contrast, research into calculating the quantity of greenhouse gases released from

Battling for science takes its toll on UN climate panel stalwarts

[KYOTO] A leading climate scientist is to leave the United Nations' expert panel, and a second is considering his future with the body. Both had been at the centre of attempts last year by US energy lobbyists to discredit the panel's conclusion that human activities are warming the planet.

Tom Wigley, a senior scientist at the National Center for Atmospheric Research in Boulder, Colorado, has decided he will no longer take part in the work of the Intergovernmental Panel on Climate Change (IPCC), which periodically reviews the world's climate change research.

His colleague Ben Santer, an atmospheric physicist at the Lawrence Livermore National Laboratory in California, says he has yet to decide whether to return to the IPCC. Santer was the main focus of a campaign last year to discredit the science of climate change (see *Nature* 381, 195; 1997).

Wigley says he feels his time is better spent "doing science, rather than reviewing it". Scientists spend too long on the review process, he says, when they should be

involved in research that will help to "prove conclusively that there is anthropogenic climate change".

Santer says a part of him now wants to steer clear of the IPCC. "It has been tremendously distressing and destroyed my family." He says that the IPCC may be better off by choosing another scientist not connected with the previous report. But he says he has learnt much from the experience of writing a report with political implications, and will not repeat his mistakes if given a second chance.

The latest IPCC report concludes that the "balance of evidence" suggests that human activity is warming the planet, and formed the basis of the Kyoto protocol agreed last week (see above). But opponents of the protocol have used the apparent discrepancy in the fact that the degree of warming from surface data is greater than that from satellite instruments to argue for a delay in action to slow down man-made climate change.

This discrepancy seems likely to continue

to be used in attempts to block or delay the protocol's ratification in the US Senate, and Wigley says that finding the reasons for it should now be a priority.

Both Wigley and Santer are also critical of a recent IPCC decision to appoint 'review editors' to liaise between scientists and authors of research, and to sign-off reports after they have been peer reviewed. Wigley says this will increase the time spent on reviews, and may deter people from volunteering to take part in the IPCC process. Santer says it will turn the task of being an IPCC author "into a full-time job".

Sir John Houghton, chairman of IPCC's science working group, is sympathetic to Wigley's decision not to be part of the IPCC. But he says that the IPCC process is important in bringing scientists together, and focusing ideas and resources on specific problems. "I hope we will continue to get leading scientists," he says. "If they get disillusioned with the IPCC process, then the quality of the reports goes down. That will be bad for science and for policy." **Ehsan Masood**

changes in land use, rice cultivation, and the clearing of forests is still in relative infancy. Most scientists did not expect these sources of emissions to be included in a protocol.

One of the first tasks — which began at a meeting of climate scientists last month — will be to 'balance' the Earth's carbon budget. At present, there is a discrepancy between the combined inventory of carbon emissions and the quantity of carbon believed to be absorbed by the atmosphere and the oceans.

Sir John Houghton, chairman of the science working group of the Intergovernmental Panel on Climate Change, says scientists will also need to define clearly the type of forest to be included in the protocol. Questions include whether emissions from managed forests ought to form part of an anthropogenic inventory — and defining the difference between a managed and a natural forest.

Similarly, researchers will need to find an accurate method of calculating average emissions and absorption of gases per unit area over a large area of forest. The problem was neatly summarized by the chairman of the Kyoto conference, Raul Estrada Ouyela: "You can't count every Christmas tree in the world."

Several research groups have already made initial estimates of the potential impact of the protocol on global temperature trends.

Their main conclusion is that, without further action, the magnitude of emissions reductions agreed is unlikely to have a major impact on global temperature rise.

According to one climate model, if all developed countries kept to their Kyoto target of reducing emissions by 5.2 per cent from 1990 levels before 2012, world temperatures would still rise by 2.11°C by 2100. This is only 0.27°C lower than the 'business as usual' scenario of world temperature rise if no intervention is taken. If developed countries were to stabilize their emissions at 1990 levels, global temperatures would rise by 2.15°C by 2100.

The data were generated by Mike Hulme, reader in climatology at the University of East Anglia. Hulme says the temperatures indicated in his calculations may not turn out to be correct, as models are subject to many assumptions and uncertainties.

Hulme says that comparing the different sets of data is more meaningful, and thinks the change in temperature if countries adhere to the protocol will be small enough to be masked by natural changes to the Earth's climate. "That is not saying Kyoto is not important... It is an important first step that will pave the way for subsequent amendments that will dig a little deeper." **Ehsan Masood**



Home at last: Japan's Ohki (left) and chairman Estrada share relief at the end of the conference.

Minister's near miss at the final curtain

[TOKYO] The need to extend the final day of the Kyoto conference meant that many participants, exhausted by rounds of all-night talks, were forced to miss their flights home and negotiate without interpreters, who left as their contracts expired at midnight on 10 December. But perhaps the biggest surprise came when the president of the conference announced his resignation and walked out of the plenary session before the finale — only to withdraw his resignation an hour later.

Hiroshi Ohki, Japan's environment minister and president of the conference, resigned from his post on the morning of 11 December and hastily left the conference hall to catch a train back to Tokyo to vote in a no-confidence motion against Prime Minister Hashimoto's cabinet. Enraged members of Japanese nongovernmental organizations are said to have pursued Ohki to Kyoto station, as well as contacting members of the parliament, to stop Ohki from boarding the train.

The parliament made a last-minute arrangement to exempt Ohki from the no-confidence vote, so he was able to resume his presidency and clinch the landmark protocol for the reduction of greenhouse gases shortly after 2 p.m. that day.

Critics claimed that the incident highlighted the lack of leadership by Japan during the conference. "The fact that the prime minister tried to call Ohki back to Tokyo on the final day of the conference reflects their [the Japanese government's] degree of commitment on this issue," says Makoto Hoshino, director of the World Wide Fund for Nature in Japan. Others, however, said that Japan should share the credit for the fact that a final compromise agreement was reached between the various conflicting points of view. **Asako Saegusa**

Faith, hope and tact needed for US ratification

[WASHINGTON] The Clinton administration will embark on the first stage of an uphill struggle to implement the Kyoto protocol when it presents its 1999 budget in February. Officials are this week working to incorporate into Clinton's budget proposal tax incentives for energy efficiency, as well as extra money for energy research, along lines suggested earlier in the year by the President's Council of Advisors on Science and Technology.

But getting these budget proposals through the Congress will be one of the easier steps required to bring the United States anywhere near its target of reducing greenhouse gas emissions to 7 per cent below their 1990 levels by 2012.

A major diplomatic effort will also be needed to bring the proposed trading scheme for emission permits — as well as some form of meaningful participation by developing countries — into

the agreement at the next meeting of parties to the climate convention, to be held in Buenos Aires next autumn. Only then will the US administration consider seeking ratification of the final treaty in the Senate.

Senator John Kerry (Democrat, Massachusetts), a strong supporter of action to cut emissions, said last week that the protocol was not ready for ratification. He would not be drawn on when it would be ready: "I don't think people should focus on ratification. We should focus on the next steps, such as the Buenos Aires meeting. We'll know when the time is right to bring this before the Senate."

Kerry points out that such timing is entirely at the discretion of the president. But he hints that the treaty may not be ratified until after 2000, when Vice-President Al Gore hopes to succeed Bill Clinton. If anyone other than Gore is elected with the treaty unratified, its prospects

would be extremely bleak.

Short of ratification, the White House can still take some executive actions — such as adjusting the government's own energy use patterns — in line with the treaty. It can also fight for, and possibly win, support for more energy research in budget negotiations with the Congress. But Senate ratification is regarded by most observers as an essential prerequisite for any break in the upward trajectory of greenhouse gas emissions in the United States.

That is because the implementation scheme proposed by Clinton on 22 October relies heavily on emitters' faith that they will win credits when a trading scheme is introduced, scheduled for 2008 (see *Nature* 389, 893; 1997). If corporations have no faith that the trading scheme will come into effect, they will not invest in cutting emissions. **Colin Macilwain**

National Institutes of Health sets up its own bioethics panel

[WASHINGTON] The US National Institutes of Health (NIH) has set up a committee to coordinate responses to new ethical issues and to anticipate future quandaries. The initiative comes at the end of a year that has seen heated public disputes about cloning and the use of placebos in AIDS-related trials in developing countries, and the breach of an embryo research ban.

The Trans-NIH Bioethics Committee was established this autumn on the orders of Harold Varmus, the NIH director, partly in response to a recommendation by an advisory panel that an NIH-wide committee should deal with ethical issues related to genetics (see *Nature* 385, 756; 1997).

But Varmus decided to make the committee's mandate broader. He felt "it was important to have substantive and frequent internal discussions on ethical, legal and social issues, so that we would be less reactive and more proactive," says Lana Skirboll, NIH deputy director for science policy, who chairs the committee.

The committee comprises senior staff representatives from each NIH institute, centre and division, and has more than 30 members. It meets privately once a month and at its three meetings so far it has discussed the capacity of the mentally ill to consent to participation in research, and medical records privacy. Future topics will include xenotransplantation and the ethical use of stored tissue samples.

A subcommittee of the new body is drafting recommendations in a 'white paper' on the confidentiality of research records and patient access to them. Expected in draft form next month, the paper will be circulated for advice and comment to scientists and patients' advocates. Ultimately, the committee hopes it will influence Congress, which is due to draft a privacy law by 1999.

But the white paper is an exception, says Skirboll. In general, she says, the committee's role is not to formulate policy but to flag issues that need attention from all parts of NIH, and "to keep ourselves on board with what's going on". Then, she says, "when we need to develop NIH-wide consensus, there is a forum in which to begin that discussion."

Skirboll cites growing public interest in bioethics as the reason why the NIH has moved now to establish an ethics committee (see *Nature* 389, 658–663, 1997).

NIH has launched separately a department of clinical bioethics at the Warren Magnuson Clinical Center on the NIH campus in Bethesda, Maryland. The department will start with a budget of \$500,000 and five permanent staff.

Meredith Wadman

Energy labs urged to boost supercomputing capability

[WASHINGTON] A new integrated strategy for high-performance computing and simulation for the national laboratories funded by the US Department of Energy has been proposed by the department's senior scientific administrator.

Ernie Moniz, the new under-secretary of energy, last week told a meeting of the Laboratory Operations Board — a group of department officials, laboratory directors and outside experts set up to oversee management reforms at the labs — that a new approach is needed to give scientists at the laboratories access to the best hardware and software for computer-intensive problems.

Moniz, who chaired the physics department at the Massachusetts Institute of Technology before joining the government last month, believes that the department's non-weapons laboratories need to take an approach to supercomputing parallel to the \$500-million-a-year Accelerated Strategic Computing Initiative (ASCI) under way at the nuclear weapons laboratories.

The directors of the non-weapons laboratories are intensely jealous of ASCI, and have been fighting without success for better access to both the hardware it will buy and the knowledge of computational science it is expected to yield.

Many computer codes used by the scientists at present, Moniz says, "are developed in a rather isolated environment, without the benefit of advanced software engineering".

Some of the department's main research tasks could benefit, Moniz says — including the simulation of combustion in car engines,

development of global climate models, hydrogeological studies for nuclear waste clean-up, and the processing of data from particle accelerators. "This kind of problem-driven approach to solving simulation problems is something the laboratories should be doing and, frankly, I don't see who else will do it," he told the board.

Moniz has asked officials to report early in the new year on the laboratories' computing and simulation needs and how they could best be met. Options could include wider use of ASCI machines for non-weapons-related work.

"I'd like to see an integrated approach across the department," says Moniz. Martha Krebs, assistant secretary for energy research, and Vic Reis, assistant secretary of defence programmes, have been "in dialogue" on the issue, he adds.

But Reis, who gets his money from the national security budget, has been reluctant to share ASCI facilities with other laboratories. Directors of the non-weapons labs have told Moniz they feel unfairly excluded from ASCI. "They have [said that], but it is changing," Moniz says. "We're not where we'd like to be yet, but I believe that Defense Programs is opening up significantly because of the changed nature of its mission."

But some officials say that Moniz may find it harder than he thinks to integrate anything across the fractious laboratory complex, as the web of connections between the laboratories and politicians makes it difficult for the energy department to administer them as a coherent group.

Colin Macilwain

New Zealand imports technology foresight

[SYDNEY] New Zealand's new government has launched a major effort to set priorities for public investment in science and technology. The exercise will use 'technology foresight' techniques, developed in Britain, Australia and Finland, to sketch out desirable futures, identify targets and develop strategies for achieving coveted goals.

Launching the project last week as one of her first acts as Prime Minister, Jenny Shipley acknowledged that New Zealand's "ability to create and use knowledge will outstrip physical resources as this country's source of wealth". New Zealand's economy has always in the past been dependent on exports of products from its fertile land. The project will be carried out through the Ministry of Research, Science and Technology, and will aim to decide public funding allocations for 2000 to 2002.



Shipley: 'knowledge is a key to wealth'.

Maurice Williamson, the minister for research, science and technology, was promoted in rank to eighth place in the reshuffled cabinet. He said New Zealand needs to "identify the tools and resources required to have a competitive advantage in the knowledge age".

Williamson's ministry provides policy advice, and is separate from funding authorities. Helen Anderson, the ministry's new chief scientific adviser, says the project followed a weekend retreat by 40 "leading thinkers" who considered possible scenarios for New Zealand up to 2010.

Peter Pockley

Researcher sues over 'fraud' sanction

[MUNICH] The University of Düsseldorf in Germany has been taken to court by a researcher in its orthopaedics department who has been stripped of his right to teach at the university following an internal inquiry into allegations of scientific fraud.

The researcher, Meinolf Goertzen, claims that the university has no right to rescind his *Habilitation*, an academic qualification required for teaching in German universities (as well as in Switzerland and Austria).

But the university is defending its actions, saying its inquiry raised serious questions about the genuineness of some of Goertzen's published experiments.

At the same time, it has welcomed new guidelines on handling cases of suspected scientific misconduct published this week by an international panel set up by the Deutsche Forschungsgemeinschaft (DFG), Germany's principal university grant-giving body (see below).

The allegations of fraud focus on a paper submitted by Goertzen in March 1994 to the *Journal of Bone and Joint Surgery (JBJS)* on the effect of gamma-irradiation on the viability of knee ligament grafts in foxhounds. (Two of the three listed co-authors deny any knowledge of the paper, while the third, Klaus-Peter Schultz, head of Goertzen's department, acknowledges 'honorary authorship'.) The paper was published the following year.

One of the figures in the paper claimed to show free sensory nerve endings reinnervating a non-irradiated graft in dogs. But soon after publication, Zdenek Halata, professor of functional anatomy at the University of Hamburg, recognized the electron micrograph as one he had taken and published in

1989 depicting stretch receptors in human ligaments. It was one of three micrographs lent to Goertzen as teaching aids.

Halata alerted the London-based journal's editor, Philip Fulford. Goertzen subsequently explained to Fulford that the picture had been sent in error, and provided another for an erratum, which was published by the journal in November 1995. But this micrograph also fell under similar suspicion.

These charges, when brought to the attention of the University of Düsseldorf, were evaluated and confirmed by its faculty of medicine's standing committee on 'good scientific practice' in early 1996. The medical faculty's standing Committee on Habilitation Affairs investigated all of Goertzen's studies on dogs, some of which had been used by Goertzen in his 1991 *Habilitation* thesis.

The investigation revealed various irregularities in Goertzen's research. These included duplicate publication of results; even Halata's electron micrograph published in Goertzen's 1995 *JBJS* paper had been published under Goertzen's name in 1994, and other micrographs had been published repeatedly with captions variously claiming to be either irradiated or non-irradiated grafts.

The number of dogs noted in the university's animal house records as being used by Goertzen in his experiments was also significantly fewer than the number referred to in his published results.

As a result of the committee's investigation, the faculty of medicine rescinded Goertzen's *Habilitation* in October 1996, along with his title of *Privatdozent* which allowed him to teach specifically at Düsseldorf University. Goertzen has challenged both

decisions in court. He declared to the court that his animal studies of 1983–94 were done both in Düsseldorf and with a company in the United States. The court refused his request that his *Privatdozent* title be reinstated pending the outcome of the case, because it judged the university's case to be very strong.

Despite efforts by Goertzen's lawyers, Fulford 'formally retracted' Goertzen's paper in an editorial published last September. One professional society of which Goertzen is a member, the German Association for Orthopaedics and Traumatology, voted to expel him shortly afterwards.

But Goertzen continues to be defended by Schultz, his head of department, who says he will continue to do so whatever the outcome of the case.

Goertzen told *Nature* that most of his experiments on dogs were carried out at private companies — which he declines to name — in the United States many years ago, and that these companies have since destroyed the records. He also said that the electron micrographs he published were meant to be illustrative, and were not essential to the scientific conclusions of his paper.

He is demanding that his case be reconsidered by a committee of experts, since the faculty Committee on Habilitation Affairs did not consist of specialists able to judge the details of his scientific work. Andreas Scheid, however, vice-dean of the medical faculty, who organized the investigation, argues: "You don't need experts to count dogs and compare photographs."

The university itself is taking the affair seriously. Its rector, Gert Kaiser, says he is investigating how Goertzen could be dismissed — not an easy matter, as Goertzen is a tenured civil servant directly employed by the state government of Nordrhein-Westfalen.

There had also been broader uncertainty, since the rights of a university to set up a panel to investigate research misconduct had been challenged by a professor of biophysics from Giessen who was himself the subject of an investigation. He claimed that it contravened the 'freedom to research' which is guaranteed in Germany's constitution. After a series of court battles the federal constitutional court has recently confirmed this right.

Scheid says he welcomes the guidelines put forward by the DFG panel of experts, which he hopes will be put in place at his university. "We had to plough hundreds of hours into the [Goertzen] case, to work out what we could and could not do legally", he says.

A standard procedure means that any future case will be easier to handle. "No one will have to start from scratch as we did," he says, adding that the panel's recommended requirements for integrity should be written into the contracts of new staff. **Alison Abbott**

Germany proposes guidelines for good practice

[MUNICH] An independent panel set up by the Deutsche Forschungsgemeinschaft (DFG) has put forward a series of proposals for ensuring good scientific practice in Germany's universities and research institutes. Institutions failing to follow the guidelines should be excluded from research funding, it says.

Although the committee stresses that none of its recommendations is new, few German universities have formal regulations for handling allegations of scientific misconduct. The DFG set up the panel in response to a recent case in

which two senior researchers were found to have systematically fabricated data over a number of years (see *Nature* **387**, 750 & **389**, 105; 1997).

In its proposals, issued this week, the committee says that universities should have clear definitions of different types of misconduct — for example, plagiarism and manipulation of data — and should make explicit where responsibility lies in a university for handling any concerns.

It also says that each institution should appoint an independent counsellor to whom a scientist aware of

scientific misconduct in their laboratory could turn, and that the DFG should appoint an independent ombudsman to consider cases of scientific misconduct. Would-be whistleblowers, or scientists who consider themselves unfairly accused, should be able to choose whether to consult the local counsellor or the DFG ombudsman for advice.

The committee further recommends that the practice of honorary authorship should be abandoned, and that all primary data on experiments should be held for a minimum of ten years. **A.A.**

Japan's research spending rises again

[TOKYO] Despite a struggling economy, overall research spending in Japan grew by 3.4 per cent in the fiscal year 1996 (which ended in March 1997), according to a survey on research and development (R&D) expenditure by the Management and Coordination Agency, a government agency that reports to the prime minister's office.

The survey, based on data provided by several thousand companies, universities and research institutes, estimates total R&D spending at ¥14.9 trillion (US\$ 115 billion). If the software industry — included for the first time in this year's survey — is added, this figure rises to ¥15.1 trillion.

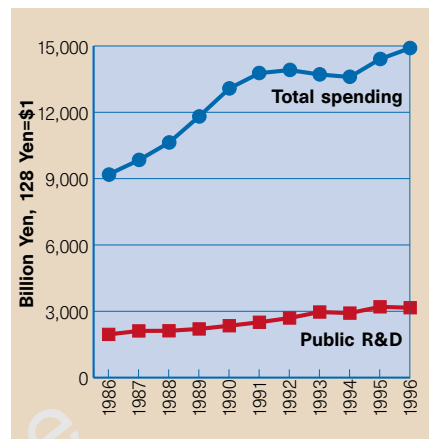
Although this year's increase is below last year's rise of 6 per cent, it is still considerably more than the growth in gross domestic product (GDP). With an estimated 3 per cent of total GDP spent on research during 1996, Japan remains well ahead of all the other large industrialized countries, whose growth rates have been below those of GDP for much of the past decade.

But although industrial R&D — excluding

software — displayed a record 5.2 per cent increase, that for higher education is estimated at a modest 1 per cent. Indeed, although spending appears to have increased markedly in private universities, public funding for university research actually declined by several percentage points. Funding at research institutes — which includes both public and private institutions — also went down, by 1.1 per cent.

Furthermore, overall public research spending decreased by some 4 per cent, according to the survey, a figure that appears to conflict with the large nominal increases in long-term government research funding budgeted for the same period (see *Nature* 385, 104; 1997). This discrepancy is partly explained by unusually generous 'supplementary budgets' for the previous year, which added considerably to a 12 per cent increase in public research spending.

As part of a ¥14 trillion government effort to stimulate the economy, supplementary budgets in 1995 included an additional ¥685 billion for research. Most of this, however,



Onward and upward: R&D expenditures have shaken off the recession of the early 1990s.

went into new buildings and facilities for universities and national institutes; extra funds for R&D in last year's supplementary budgets amounted to only ¥155 billion.

The large increase in private expenditures revealed by this year's survey appears to confirm claims that the political objective of the 'Basic Law' was aimed less at raising public research spending than at reinvigorating private-sector R&D. After periods of sustained growth during the 1980s, companies had been cutting their research budgets in the early 1990s because of the recession.

But since last year, industrial R&D has been rising again. This trend appears to be confirmed by a recent survey of some 200 large corporations by the *Nikkan Kogyo Shimbun*, a daily newspaper for business and technology, which found growth rates for 1997 of between 2 and 10 per cent for all industries except petroleum refining. Growth in industrial R&D has been most marked in the electronics, telecommunications and transport sectors. Total R&D in software was estimated at only ¥177 billion, a sum that is below R&D spending by some individual US companies.

According to Shinichi Kobayashi, a science policy analyst at the University for Electro-Communications near Tokyo, R&D expenditure in small and medium-sized enterprises is inadequately measured, and as a result the data provided by the survey may underestimate structural changes in the Japanese economy.

In particular, analysis of inter-company R&D funding, as measured by the annual survey, reveals a widening gap between the total amount received by industry and that paid out for research, leaving a growing proportion of funds spent on extramural R&D unaccounted for. According to Kobayashi, this suggests a trend towards out-sourcing of R&D to small companies that does not show up in the survey data.

Robert Triendl

Inquiry looks into Indian cancer deaths

[NEW DELHI] An inquiry is being set up to investigate allegations that medical treatment was deliberately withheld from 1,107 Indian women with uterine cervical dysplasias, even though it was known that some of the lesions could become cancerous. The seven-member inquiry committee will be headed by a retired judge.

The women, who were attending gynaecology wards in six hospitals, were part of a study on cervical cancer, but had not given written consent to take part in the experiment. Rather than being treated, they were registered for a long-term follow-up, during which 69 women 'progressed to malignancy' and had to undergo cancer treatment or have the uterus removed. Two women died during radiation treatment. Investigators do not know what happened to ten others with advanced dysplasias who failed to report for the follow-up.

The study, conducted between 1976 and 1988 by the Indian Council of Medical Research (ICMR), was intended to identify risk factors in the transformation of dysplasias into cervical cancer, the biggest killer among cancers in Indian women.

Usha Luthra, ICMR's then deputy chief and the project's principal investigator, maintains that the women were "verbally" informed, as written consent was not mandatory at the time. "Ethical guidelines keep changing. If I had to go back to the 1970s I would repeat the study exactly the same way," she says. She says the study

helped India to evolve screening guidelines for a national cancer control programme.

But some doctors and women's groups are questioning the ethics of the study in the absence of documented proof that the women — mostly poor and illiterate — were made aware that their lesions could develop into cancer without treatment. Saheli, a prominent women's group, has appealed to the National Human Rights Organization for "stringent actions against those responsible for the barbaric tests".

Vulimuri Ramalingaswami, who headed ICMR between 1978 and 1985, argues that the study should be viewed in the context of the ethical standards of the time (the ICMR had no guidelines until 1980). "When it was found that moderate and severe dysplasias more often turned into cancer, such women were taken off the study and put on treatment," he says.

But N. P. Gupta, formerly deputy director general of ICMR, describes the study as inhuman. "I did not know that such a study was going on. We are all criminals." G. V. Satyavati, the immediate past head of ICMR, ordered a probe by an independent committee before she retired in August.

Although the controversial study was closed nine years ago, it is being highlighted now to ensure that ICMR's guidelines — which are being revised by a committee headed by the former chief justice of the supreme Court — are made tougher than those issued in 1980.

K. S. Jayaraman

Royal Greenwich Observatory bosses lose bid for support

[LONDON] Prospects that the Royal Greenwich Observatory, based in Cambridge, will eventually return to its historical roots in southeast London increased last week. The Particle Physics and Astronomy Research Council (PPARC) declined to provide financial support for a bid by the observatory's managers to remain in Cambridge as an independent company limited by guarantee (see *Nature* 390, 540; 1997).

A statement by the managers said that, having failed in their bid for research council funding for the observatory's work with universities and foreign telescope facilities, they will "seek sponsorship or support" for such work "to continue to the next millennium". But the University of Cambridge, which PPARC is hoping to persuade to purchase the present observatory building, has also declined to provide backing for the proposed independent company.

PPARC concluded that investment in the company was "not appropriate" and is to explore how the observatory might be incorporated into the National Maritime Museum in Greenwich.

Italy's research chiefs hit out at budget cuts

[MUNICH] The heads of the 15 research committees of Italy's National Research Council (CNR), which advise the council's president and distribute its relatively small pot of research funds, have condemned the government's proposed 1998 CNR budget, which halves the agency's funding for research (excluding salaries and overheads).

The money that the committees allocated to university researchers, which was set last year at IL65 billion (US\$37 million), has been reduced to zero, possibly in anticipation of a new bill, not yet passed by parliament, that would end CNR's role as a university grant agency. In addition, the money for 'bottom-up' research projects in CNR institutes has been reduced by 30 per cent in the budget proposal. Funds for strategic research projects have been halved. The budget must be approved by parliament before the end of the year.

Europe's space centres 'must work together'

[MUNICH] National space research centres in the member states of the European Union should cooperate more closely, according to Walter Kröll, head of Germany's national air and space research centre (DLR). Speaking at

the DLR's annual press conference in Bonn last week, Kröll said that successful competition with the United States could be achieved only by a joint effort between Europe's industries and the seven national space research centres. He proposed that the centres should join together in a European Space Research Association with strategically coordinated research activities.

Asia-Pacific countries to pool quake knowledge

[SAN FRANCISCO] Earthquake research groups in Australia, China, Japan and the United States have agreed to collaborate under the umbrella of the Asia-Pacific Economic Cooperation forum (APEC) in an effort to develop realistic numerical simulation models of earthquake generation. Data from earthquake observations would be assimilated into such models.

The initiative is being coordinated by Peter Mora, director of the University of Queensland's Advanced Centre for Earthquake Studies. He says that APEC's member 'economies' are subject to a large number of the world's earthquakes.

The idea of the initiative is to provide mutual support for the activities of complementary programmes in participating APEC countries. "We want to put these together to maximize the amount

of science that comes out," says Mora. Working groups and an international science board will be set up next year and the initiative "should be in full swing in 1999".

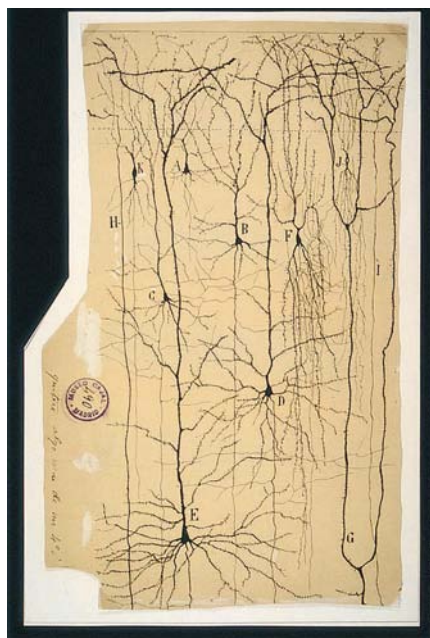
Missile hits Japanese equipment in Utah

[WASHINGTON] US Air Force officials are still trying to explain how an unarmed Cruise missile fired last week from a B-52 bomber over a testing ground in the Utah desert accidentally struck and damaged two trailers full of computers and other equipment used by a Japanese cosmic-ray observatory.

No one was hurt in the incident, which occurred on 10 December at the army's Dugway Proving Ground west of Salt Lake City. The telescope array, operated by the University of Tokyo, is one of several existing or proposed cosmic-ray experiments in the area around Dugway, which also has storage facilities for biological and chemical weapons. An investigation is under way.

Shuttle flight will honour neuroanatomist Cajal

[MADRID] The grand finale of the US Decade of the Brain will be a 17-day shuttle flight in April next year devoted entirely to neuroscience experiments. It will be dedicated to Santiago Ramón y Cajal, the



Spanish neuroanatomist who won the Nobel prize for medicine in 1906 jointly with Camillo Golgi for identifying neurons as individual entities.

Cajal was also an artist. Eight of his neuroanatomical drawings (above) from the collection housed at the Cajal Institute for Neurosciences in Madrid, along with some of his original microscope slides, will fly with the mission. They will be described by

astronaut Dave Williams in a live broadcast from the shuttle during its flight.

Each of the eight drawings represents one of the scientific areas under study on the shuttle flight. These include sleep, development and the balance system. Around 50 experiments will be carried out by the astronauts, who will also act as subjects in some of them.

Pioneer herpesvirus experiment on the way

[WASHINGTON] Plans are under development for what may be the first gene therapy experiment using a herpesvirus vector. Its author, Bernard Roizman of the University of Chicago, last week described the biology of herpesvirus to the US National Institutes of Health's Recombinant DNA Advisory Committee (RAC), which had asked for a briefing in anticipation of a protocol he is expected to submit soon.

The session was the first meeting of the newly reformulated RAC, which in October became advisory in function, without the power to approve specific protocols. Officials said the two-day session on gene therapy technologies was intended to be "forward looking, before these technologies are upon us". The meeting also heard about lentivirus vectors, gene 'shuffling' and artificial human chromosomes.

Time for change in Portuguese science

Sir — There is an almost perfect positive relationship between scientific output and gross domestic product (GDP) in Western Europe (Fig. 1). But two countries — Ireland and Portugal — spoil the relationship.

Ireland, wisely, shows intense scientific activity, unlike Portugal, which publishes three times less than would be expected for its GDP. The general relationship between number of papers published and GDP is also true for the social sciences, and the arts and humanities (not shown), and the gap between observed and expected output for Portugal is just as wide.

The situation in Portugal requires immediate action if the country is to be ready to face the next century in an increasingly competitive world, where science and technology provide the driving force. Scientific output is almost exclusively correlated to spending on research and development (R&D) ($r = 0.949$ for the countries in Fig. 1). Portugal spends only 0.60 per cent of GDP on R&D, whereas the rest of Western Europe spends on average 1.78 per cent of GDP. At the present rate of increase in spending on R&D, it will be 50 years before Portugal reaches the current European average.

More funds need to be invested in Portuguese science and higher education. But a consistent policy and pooling of existing resources is also required. Small groups with good scientific potential should be brought together to create a critical mass and provided with a good scientific infrastructure coupled with regular evaluation of achievement. Longer-term funding should be provided instead of the current system based on irregular calls for project applications. To be fair to the present government, steps have been taken in the right direction, with the evaluation last year of research units by international experts and the introduction of some basic long-term funding. The government has also, correctly, put emphasis on pre-primary and up to secondary school level education. It is now time to concentrate on higher education and research, paving the way for future generations.

A dramatic change in the university system, where most Portuguese science is carried out, is also required. Learning science in the universities has been undermined by chronic low funding (the annual cost of an undergraduate student in Portugal is 2.5 times less than in the United Kingdom), lack of basic equipment, ludicrous financial and accountancy rules, poor libraries and excessive teaching loads for lecturers. Universities have started some

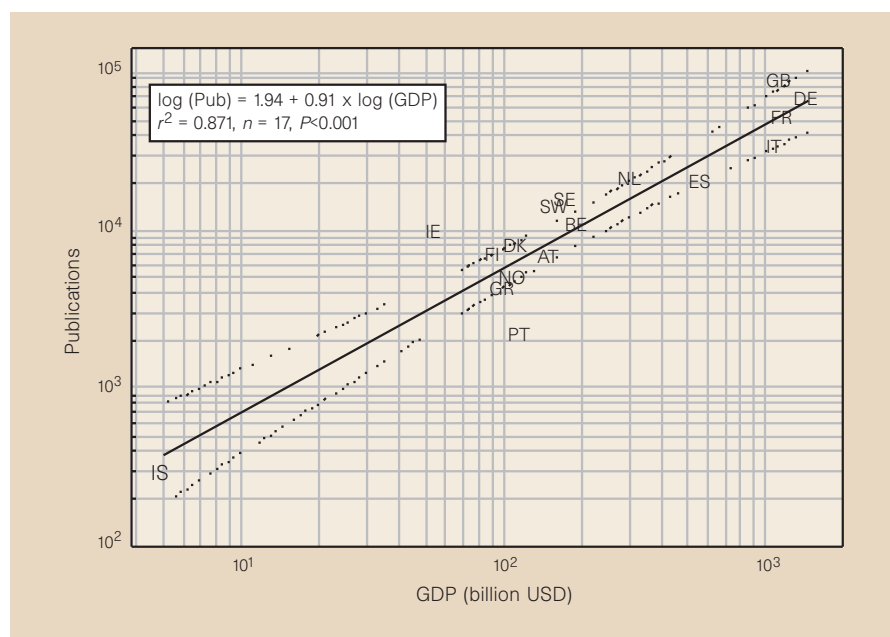


Figure 1 Scientific output in 1996 in Western Europe as a function of GDP in 1995. Dotted lines are 95% confidence limits of regression line. Data from: *Science Citation Index* (Institute of Scientific Information) and *The World Fact Book* (US Central Intelligence Agency). AT, Austria; BE, Belgium; DE, Germany; DK, Denmark; ES, Spain; FI, Finland; FR, France; GB, United Kingdom; GR, Greece; IE, Ireland; IS, Iceland; IT, Italy; NL, Netherlands; NO, Norway; PT, Portugal; SE, Sweden; SW, Switzerland.

sort of self-evaluation but have been showing little enthusiasm in the process.

There is also an indescribable and unique demagogic university autonomy system in which undergraduate students and teaching assistants (PhD students) hold up to 70 per cent of the vote for the election of rectors and heads of faculty. Lecturers and professors hold only 20 per cent of the vote, the other 10 per cent being held by administrative and support staff.

In this climate, planning is almost impossible, irrespective of the amount

of funding. It is time for change. Otherwise, Portugal will continue for decades at the tail of scientific and academic achievement. Five hundred years ago, technology gave the Portuguese advantage over other countries, something that we like to boast about.

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Quid pro quo

Sir — The Dearing proposals on education in the United Kingdom, although not unreasonable in themselves, carry a serious threat to the recruitment of able postgraduate students working for higher degrees. Not only do such students represent the next generation of research workers but their work contributes very significantly to research in progress.

Yet their career prospects are not such as to put them in a position to repay the debt they will have accumulated to cover the cost of their undergraduate education; postgraduate education involves a further period of relative penury with no guaranteed prospects of a tenured appointment when completed.

It would be in the interests of Britain to cancel the debts of such students on their gaining a PhD in an approved field of study within a reasonable time limit, and perhaps also of those who serve a satisfactory apprenticeship in medicine or teaching. This would not be very costly because the numbers are relatively small, and it would act as a spur to recruitment and the completion of their theses.

Perhaps also the United Kingdom should think of designating some of its universities as exclusively postgraduate institutes along the lines of Princeton.

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Plant cyclic AMP comes in from the cold

Anthony J. Trewavas

Whether or not plant cells contain the growth factor cyclic AMP has been a source of controversy for over 25 years. Now, characterization of an enzyme that produces cAMP and mediates the action of the plant hormone auxin marks a decisive defeat for the majority view.

Cyclic AMP is a key growth factor and signalling molecule. Produced by the enzyme adenylyl cyclase, it mediates the action of many other growth factors and hormones. Yet despite the numerous growth regulators in plant cells — and an evident need for growth regulation during development — it has been dogma for 25 years that higher plants do not possess adenylyl cyclase, do not use cAMP and are, thus, unique among living organisms.

Bitter controversy in the 1970s surrounded the initial claims that cAMP is found in plant cells. Two camps rapidly separated — sceptics who could find only vanishingly small quantities, and believers who reported biologically meaningful micromolar concentrations, albeit with weaker technology. At meetings to resolve the issue, heated and vitriolic exchanges often took place between believers and sceptics: “Does Dr ... really choose to join those who incorrectly report the presence of cAMP in plant tissues?” is a statement I recall from one such altercation. Made by one of the most powerful figures in US plant physiology at the time, such attitudes rapidly relegated believers to obscure journals and the likelihood of failed tenure or promotion expectations, as opinion swung into line behind the dominant hierarchy. The dogma that plants contained either no cAMP or, at best, biologically irrelevant concentrations, rapidly assumed the rule of law and textbook status^{1,2}. Believers, few in number, would make occasional forays to semi-respectable journals. But they were met with repulse by the sceptics, who again reported that cAMP, if it was there, “... was below the detection limit ...” of very sensitive radio-immune assays³. What the believers lacked — which would have resolved the issue firmly in their favour — was a convincing demonstration that plant cells contain a functioning adenylyl cyclase.

Over the past decade, however, cracks have appeared in the sceptical edifice⁴: sequences similar to proteins that bind cAMP and regulate gene expression have turned up in plant libraries. Moreover, in

guard cells (which regulate gas exchange into and out of the leaf), potassium channels have been found to respond to cAMP-dependent phosphorylation. But perhaps the most telling information originated with plant phosphoinositide studies. Attempts to measure the concentrations of phosphatidylinositol mono- and bisphosphates — the precursors of inositol-1,4,5-trisphosphate, $\text{Ins}(1,4,5)\text{P}_3$ — could detect only vanishingly small quantities in plant tissues. However, using loaded, caged $\text{Ins}(1,4,5)\text{P}_3$ to release the compound in plant cells, activity was found in guard cells, protoplasts from

etiolated leaves, and growing pollen tubes. Not only did $\text{Ins}(1,4,5)\text{P}_3$ initiate the release of intracellular Ca^{2+} , but it caused direct physiological changes^{5,6}. These plant cells respond easily to many signals, and all have now been shown to have functional phosphoinositide systems.

In many plant tissues, massive downregulation of signalling constituents is thought to accompany tissue maturation. Could downregulation of adenylyl cyclase account for the missing cAMP^{5,6}? On page 698 of this issue, Ichikawa *et al.*⁷ have decisively answered this question. A team led by Rick Walden has not only identified adenylyl cyclase in regeneration of tobacco protoplasts but, by overexpression in bacteria and complementation in yeast, has left no doubt as to its biological activity. When they treated protoplasts with forskolin (which activates adenylyl cyclase) or cAMP, protoplast regeneration occurred in the absence of the endogenous plant growth regulator auxin (indoleacetic acid). So cAMP is clearly part of the auxin signal-transduction chain.

The growth of stems, roots, flowers and fruits is controlled by a variety of growth factors, among them, auxin. In concert with other growth regulators (notably the adenine-based cytokinins), auxin also regulates the growth and division of cultured cells, protoplast regeneration and callus

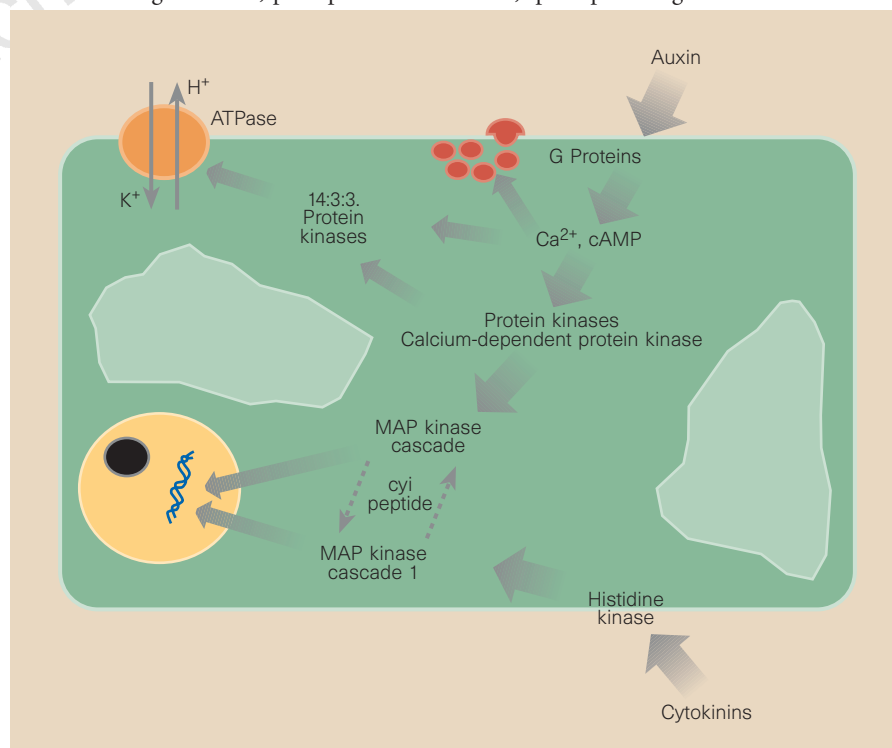


Figure 1 Transduction sequences of auxins and cytokinins. Ichikawa *et al.*⁷ have laid to rest the debate about whether plant cells contain cyclic AMP, by identifying functional adenylyl cyclase (the enzyme that synthesizes cAMP) and showing that it can mediate the actions of the plant hormone auxin. The growth-regulating effect of auxin is pre-eminent, and the main role of cytokinins is to attenuate this growth. So whereas auxin can regulate growth in the absence of other growth regulators, cytokinins do so only in the presence of auxin. The cyi peptide is suggested to join the two mitogen-activated protein (MAP) kinase signal-transduction cascades¹¹. The diagram includes only some of the known transduction information.

formation. With callus of some species, high ratios of auxin to cytokinin in the medium induce the formation of roots, whereas low ratios induce shoot formation.

Now that Ichikawa *et al.* have shown that auxin signals are transduced through cAMP (and, from other studies⁸, probably through cytosolic Ca^{2+} as well), we can see that auxin follows a familiar transduction pattern. Auxin signals activate protein kinases, which regulate the electrogenic ($\text{H}^+ + \text{K}^+$)ATPase and gene expression through a mitogen-activated protein (MAP) kinase cascade (Fig. 1). No doubt protein kinase A will find a prominent place in this sequence. And with regulation of cell-wall vesicle secretion by cytosolic Ca^{2+} , enabling increased growth of the cell wall, the pieces of the growth jigsaw are beginning to fall into place.

In the absence of other growth factors, auxin induces highly polarized cell growth, exemplified by elongation of cells from the coleoptile (the sheath that protects growing shoot tips). Overexpression of cytokinins in tobacco reduces stem height and increases stem thickness. Cytokinins, the receptor for which is now thought to be a histidine kinase⁹, seem to be transduced only through MAP kinase cascades, with no involvement of Ca^{2+} or cAMP. In this case, then, cytokinins simply attenuate auxin-generated growth, and redirect cell-growth polarity to a more isodiametric shape.

Cell shape often underpins overall tissue form: a high ratio of auxin to cytokinin will encourage the regeneration of long, thin

tissues (such as adventitious and lateral roots) from callus, whereas a low auxin to cytokinin ratio ensures the regeneration of the thicker, more isodiametric tissue forms that we associate with the shoot. Recently identified cyi peptides¹⁰ may mediate the cytokinin attenuation and induce the expression of cyclins and cyclin kinases¹¹. These enable plant cells to grow and divide in the presence of both auxin and cytokinin.

Knowledge of the transduction sequence of auxin brings us much closer to the holy grail of understanding morphogenesis — tissue form. But the solution to one puzzle highlights equally pressing questions. For example, other growth factors (such as gibberellins, abscisins and ethylene) also control the shape and division of plant cells. Will these regulate growth and tissue form through adenylyl cyclase and Ca^{2+} , or will they, like cytokinin, simply attenuate the effects of the master controller, auxin? □

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camel, but whether the one-humped (Arabian) camel or dromedary is descended from the same or a different ancestor is a moot point. The Bactrian camel differs from its Arabian cousin not only in having two humps, but also in its shorter legs and stockier build. Moreover, differences in bone morphology underlie the different shapes of humps in Bactrian and Arabian camels, lending support to the idea that these camels are different species. Nonetheless, fertile offspring can result from crossing these two varieties. The offspring have a single, somewhat indented, long hump and are even said to exhibit hybrid vigour; but continued crossbreeding rapidly results in loss of fertility. Together, these findings suggest that the Bactrian and Arabian camels are different species with a separate ancestry.

As Peters and von den Dreisch point out², identifying the earliest evidence of any domestic animal in the archaeological record is fraught with problems. It can be difficult to determine if artistic representations or bone remains (which are often fragmentary) represent domestic or wild camels, particularly as the earliest domesticates were anatomically very similar to their wild ancestors. Moreover, the archaeological context of the finds must be scrupulously examined. Animal remains from sites overlain with the remnants of later occupations can be unreliable as records of early camel domestication because of possible contamination by younger material. Direct radio-carbon dating of animal remains is essential.

Applying these criteria to their analysis, the authors find that many records of domestic Bactrian camels from central and southern Asia in the third or fourth millennium BC are unreliable. They conclude that the earliest acceptable records are from Bronze Age sites (about 2500–2200 BC) in the Kopet Dagh foothills of Turkmenistan and northeastern Iran, where finds of camel bones are associated with clay figurines of camels with nose-peggs, a clear sign of domestication. Even so, the authors reject the popular hypothesis that the inhabitants of this area had a major

Animal domestication

Remedies for windy camels

Adrian M. Lister

If your camel suffers from violent wind, “the whole belly is swollen ... the ears are put together, the teeth are firmly closed”. The remedy, according to a Chinese manual on camel husbandry from the twelfth century AD, includes “powder of centipedes, beans soaked in wine, acupuncture behind the ears, and the letting of a great quantity of blood”. This manuscript, recently translated by Herbert Franke¹, is the oldest surviving compendium of its kind, describing a total of 48 afflictions of camels and their remedies (Fig. 1). Camel husbandry clearly has a long history in China — but when, where, from what, by whom and for what purpose was the camel first domesticated? These are the questions addressed by Peters and von den Dreisch² in a new and thorough analysis of the origins of the two-humped Bactrian camel (including discussion of the ancient camel manual) published in the *Journal of Zoology*.

The camel family (Camelidae) originated in the New World, but by the Middle

Pliocene (about three million years ago) camels had migrated into the Old World across the Bering Land Bridge. The two-humped wild species, *Camelus ferus*, is generally considered to be the ancestor of the living, domestic, two-humped (Bactrian)

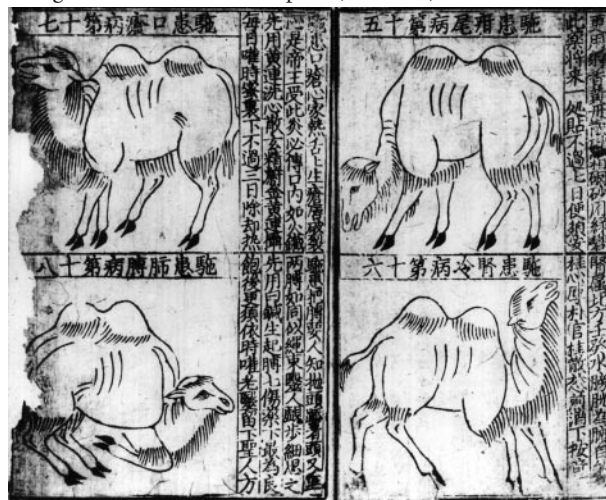


Figure 1 Two pages reproduced from a twelfth-century Chinese manual on camel husbandry, the *Fan-mu tsuan yen-fang*. The translations of the writing above the drawings are as follows. The camel suffers from eruptions on the tail (top right); the camel suffers from the cold of the kidneys (bottom right); the camel suffers from ulcers in the mouth (top left); the camel suffers from joint infection (bottom left). (From ref. 2.)

role in the domestication process. Their reasoning is simple — having scrutinized the Mesolithic and Neolithic bone record covering the preceding several millennia, they find no evidence that the wild camel, *C. ferus*, was present in these regions. Perforce, they argue, this cannot have been the area of original domestication, which they predict occurred farther to the east, perhaps in Kazakhstan, northern Mongolia or northern China, areas clearly inhabited by *C. ferus*. Even in China — despite the detailed understanding of camels revealed by the twelfth century AD medical manual — there is no direct evidence of the domestication process itself. The earliest surviving mention of Bactrian camels in the Chinese literature is as late as the fourth century BC, long after the original domestication event.

How, then, did the human–camel relationship change from hunting to domestication? Contrary to their image as long-distance nomads, in the wild state, camels have a strong attachment to their home area. It is possible that, especially during droughts, camels and humans would have been

brought into close contact, as both sought the same watering places. The authors postulate that it seems most likely that camels were first kept for their meat — they grow rapidly and can inhabit arid areas where the vegetation is insufficient to support cattle or sheep. Soon people learned to exploit the benefits of wool and dung — camels shed copious quantities of high-quality wool, especially in the spring moult, and their dung has excellent burning properties, producing a hotter, cleaner flame than wood (of value when fires are built in tents). Peters and von den Dreisch suggest that the use of camels for carrying heavy loads or as a means of transport came later.

This exemplary study of a neglected animal illustrates the rigorous approach necessary for appraising the origins of domestication in any species and, in so doing, demonstrates the maturity and importance of archaeozoology as a science. □

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Quantum chromodynamics

Colour takes the field

Frank Wilczek

Sometimes discoveries in physics come as sudden revelations: the existence of a new kind of particle, or of a new phase of matter. But sometimes profound results take shape gradually, appearing first as suggestive but not unique interpretations of limited data, becoming fully realized only as they are recognized in different contexts and made quantitative. The phenomenon of colour coherence is of the latter type. Its observation^{1,2} is a very remarkable result, showing in a dramatic way the unity and predictive power of fundamental physical theories; yet it has come upon us so gradually that it has gone almost unremarked.

To set the stage, let me briefly recall the close analogies between quantum electrodynamics (QED) and quantum chromodynamics (QCD), the theory of the ‘strong’ force which binds atomic nuclei and governs most high-energy particle interactions. Both theories are based on the concept that fields respond to the presence and motion of charges. In QED, the electromagnetic field responds to electric charges. In QCD a more complicated system of eight different fields responds to the presence and motion of three different kinds of charge, somewhat inappropriately called colours (‘red’, ‘green’ and ‘blue’). Although the mathematics of QCD is more intricate — and thus, to aficionados, more symmetrical, beautiful and challenging — than that of QED, the

basic interactions postulated in the two theories are profoundly similar.

Electrons and positrons (among other particles) carry electric charge, and quarks and antiquarks are their colour counterparts. Photons are the corpuscles of the electromagnetic field of QED; the eight colour gluons are their analogues in QCD. Gluons are now routinely observed as individual particles, signalled by the jets of energetic particles they leave in their wake. But amid all the close analogies, there is one important difference between QED and QCD, which is responsible for their very different dynamics: whereas the photon is electrically

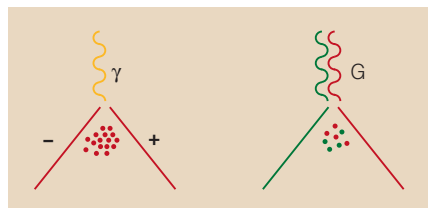


Figure 1 Addition of colours. These two types of three-jet event are produced by the annihilation of electrons and positrons. When a photon (γ) accompanies a quark and antiquark, the quark and antiquark have equal and opposite colour charge (here, red). When there is a gluon (G) instead, the quark and antiquark have different, but not opposite, colour charges. The coherently reinforced colour field in the first case creates far more particles than the incoherent fields in the second.



100 YEARS AGO

Different minds place different estimates on the intellectual accomplishments of the past half-century. In ordinary conversation the men of the mart will point to an Eiffel tower, a suspension bridge, a continental express train, a man-of-war, or an Atlantic cable. But in a discourse recently delivered in commemoration of the jubilee of the Sheffield Scientific School of Yale University, President Gilman remarked that perhaps the greatest triumphs of the intellect during the last half-century are these five contributions to human knowledge: the establishment of the principles of evolution; the establishment of the principle of the conservation of energy; the development of mathematical science and its application to physics, mechanics, electricity and astronomy; the development of spectrum analysis and the consequent discoveries respecting light and electricity; and the discovery of the nature and functions of bacteria, and of their influence, for weal or woe, upon living organisms.

From *Nature* 16 December 1897.

50 YEARS AGO

In 1914 Walter Jones began the preface to his monograph on nucleic acids with the words, “The nucleic acids constitute what is possibly the best understood field of Physiological Chemistry...” That was then a tenable point of view; but twenty years later it would have been absurd. There are fashions in everything, including biochemistry, and nucleic acids went largely out of fashion or were swamped by the growth of our knowledge of proteins, oxidation mechanisms and the other processes that characterized the development of biochemistry in the 1930’s. The boom-slump-boom cycle is, however, not confined to economics, and a nucleic acid boom is now upon us. Much new knowledge of the intrinsic properties and biological behaviour of the nucleic acids has been gained during the past few years; but there seems to have been a disproportionate flood of review articles, conferences and symposia. Stocktaking is valuable, but what is now needed in cytochemistry is more stock rather than more surveying.

N.W. Pirie

From *Nature* 20 December 1947.

neutral, the gluons themselves carry colour charges (Fig. 1).

The soul of QED is the electromagnetic field. When Faraday and Maxwell proposed this field, their contemporaries found it very abstract and mysterious. But as antennas were devised that danced to its tune, the electromagnetic field came to seem undeniably real and almost tangible.

Can one devise antennas for the colour fields of the strong interaction? It is not possible to do this directly, because the wavelengths involved are ridiculously small — 10^{-14} cm and below, much smaller than X-rays or even ordinary gamma-rays. Fortunately, colour coherence provides an indirect path to the goal.

What follows is an example of colour coherence which makes an especially vivid impression, owing to its use of photons and gluons in contrast.

Usually, when a high-energy electron and its antiparticle, a positron, annihilate, two narrow sprays of particles (jets) emerge, produced by a short-lived quark–antiquark pair. Some slow-moving particles are also left behind. The number of particles produced at a given time and place measures the strength of the colour field there, much as an antenna's response measures the strength of electromagnetic fields. This multiplicity is roughly proportional to the intensity, or energy, of the field, which grows as the square of its magnitude. So, in effect, the slow-moving particles provide a peculiar form of antenna — one that is fairly crude, but unique in its sensitivity to exceedingly short-wavelength colour fields, ready-made and very cheap.

This antenna has been used to distinguish between coherent and incoherent fields, which are produced in two slightly rarer cases of electron–positron collision. In about ten per cent of the events, there is a third jet containing several strongly interacting particles. This third jet signals the radiation of a colour gluon. Still more rarely, in fewer than one per cent of the events, an energetic photon accompanies the quark and antiquark (Fig. 1). Fortunately, as millions of electron–positron annihilation events have been studied, there are many examples of both rare types available for analysis.

Colour charge, like electric charge, is conserved. Because the original electron and positron had zero colour charge, the colour charges of the annihilation products must add up to zero. When the quark and antiquark are accompanied by a photon, their colour charges must be equal and opposite, as the photon is colour neutral. The positive charge (red, say) creates a red colour field that points away from it, whereas the field of the negative red charge points towards it. In the region between, these two contributions reinforce each other — the fields are coherent. But when the quark and antiquark are accompanied by a gluon, their colour

charges are generally of completely different types, not equal and opposite, and the fields between them do not reinforce each other.

QCD predicts, therefore, that more particles will be produced between the quark and antiquark jets when they are accompanied by a photon, as compared to when they are accompanied by a gluon jet. Just such an effect is observed. The difference is about a factor of two, which agrees quite well with rigorous theoretical calculations³.

Other, more complex colour coherence effects are seen in other types of event. For example, in collisions of particles other than electrons and positrons — such as quarks and antiquarks⁴, or quarks and electrons —

the colour fields of the initial particles can reinforce, or cancel, the colour fields of the particles produced.

As these patterns are mapped out, colour fields are becoming an ever more tangible aspect of reality. The recent accumulation of definitive results from a variety of experiments makes this an appropriate time to declare a triumph. □

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Photonics

Frozen light

Sajeev John

Electromagnetism is the fundamental mediator of all interactions in atomic physics and condensed-matter physics — in other words, the force that governs the structure of ordinary matter. It is rare to see an entirely new electromagnetic phenomenon, but Diederik Wiersma and colleagues report one on page 671 of this issue¹. In carefully prepared semiconductor

powders, Wiersma *et al.* have shown that light can be forced to stand still through the process of multiple scattering and wave interference. This experiment will lead to applications in optical data processing, spectroscopy and laser physics.

Our understanding and manipulation of electromagnetic waves has a long and rich history. The propagation of electromagnetic

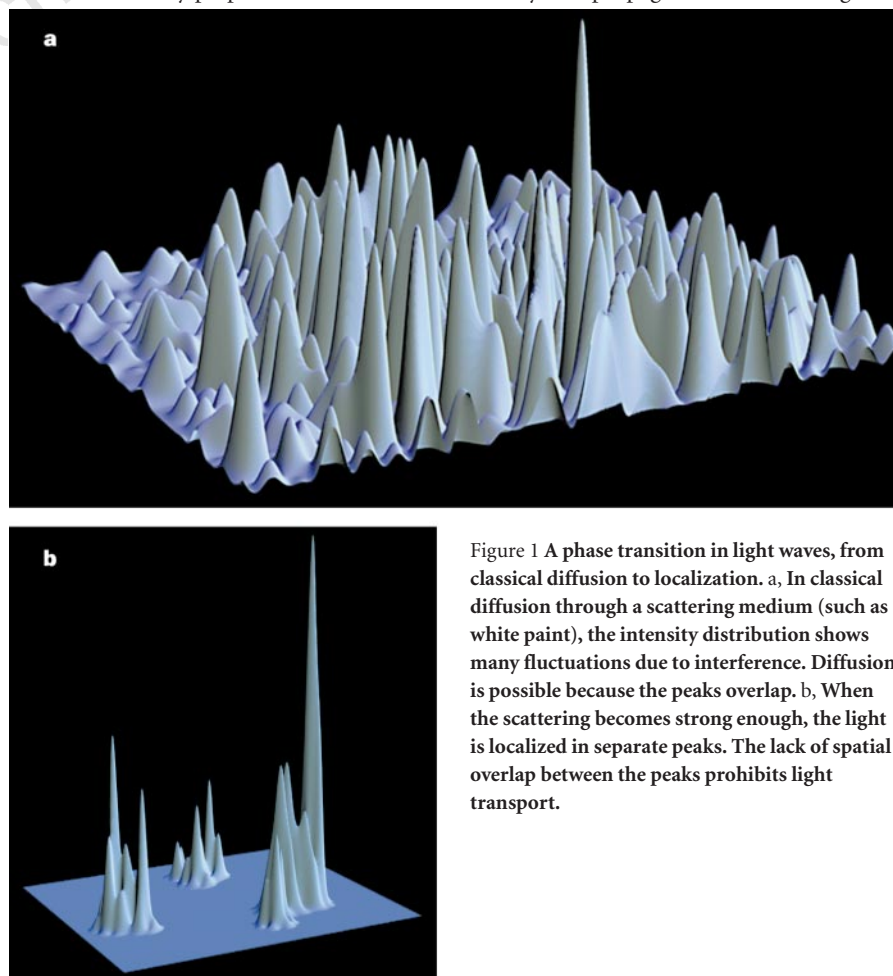


Figure 1 A phase transition in light waves, from classical diffusion to localization. a, In classical diffusion through a scattering medium (such as white paint), the intensity distribution shows many fluctuations due to interference. Diffusion is possible because the peaks overlap. b, When the scattering becomes strong enough, the light is localized in separate peaks. The lack of spatial overlap between the peaks prohibits light transport.

waves was predicted by James Clerk Maxwell in the nineteenth century, and Maxwell's equations are one of the pillars of modern physics. But the localization of light waves was predicted²⁻⁴ only in the 1980s, and confirmatory experiments (first in the microwave spectrum^{5,6}, and now in the near-visible spectrum¹) are milestones in our endeavour to create materials that mould the flow of light.

We all experience the effects of multiple scattering when it becomes dark on a cloudy day. Light from the Sun scatters many times from water droplets, following a tortuous diffusion path before reaching the ground. The distance that light travels within the cloud before being scattered into a random direction is called the mean free path. The effect of multiple light scattering is that the total amount of light transmitted through the cloud is reduced by a factor of the ratio of the cloud thickness to the transport mean free path. The rest comes back out of the other side, which is why clouds (and powders) look white.

Multiple scattering of light also takes place in human tissue. Here, the transport mean free path for infrared light of one micrometre wavelength is about a millimetre. Understanding the diffusive propagation of light in tissue is already paving the way to infrared imaging, a cheap and safe alternative to X-rays and nuclear magnetic resonance in diagnostic imaging of tumours⁷.

But neither clouds nor human tissue can scatter light strongly enough to cause localization. To achieve that, Wiersma *et al.* slowly ground samples of the semiconductor gallium arsenide to create a fine powder with a high refractive index, which scatters light roughly a thousand times more strongly than tissue. In their experiment, the transport mean free path is about the size of the wavelength of light — a regime of scattering never before attained. In this regime, the photons no longer travel like billiard balls bouncing randomly through a maze. Instead, there are strong interference effects, due to the wave-like nature of the photon, which severely impede diffusion (Fig. 1). This becomes evident in the transmission properties of the medium: for grain sizes near the onset of localization, the transmission coefficient falls off as the square of the optical depth (the ratio of mean free path to sample thickness); and when localization occurs, transmission falls off exponentially.

Unlike electrons in a semiconductor, which are always conserved in number, photons are readily absorbed by matter. This complicates the interpretation of experiments, because absorption alone can lead to exponential decay of the transmitted light intensity. In order to separate the effects of absorption from true localization, Wiersma *et al.*¹ varied the temperature of

their semiconductor powders over a range of about 500 K. This leads to characteristic variations in the wavelength of the material's optical absorption edge, allowing localization and absorption to be distinguished. To further confirm that wave interference causes the localization transition, the authors compared their results with coherent back-scattering experiments^{8,9} on the same samples, which directly measure the interference between time-reversed optical paths.

The history of light localization has unfolded in a manner that is almost the reverse of electron ('Anderson') localization. In the case of electrons, the invention of the semiconductor preceded considerations of localization. In the case of photons, considerations of localization are a driving force in the development of photonic band-gap materials^{10,11}, the photonic analogue of the semiconductor. These materials consist of a periodic array of scatterers with a lattice constant comparable to the wavelength of light. Photonic band-gap materials have important technological applications — in the development of micro-lasers and optical transistors, for example — because they can coherently localize light when disorder is introduced (either by structural defects or by doping the material with resonant atoms or molecules).

Powders are much easier to make than photonic band-gap materials, and the localization length in a powder is much longer: the photon may propagate hundreds of wavelengths before realizing that it is completely trapped. That may be very useful for

cooperative effects involving photons and atoms, such as laser action.

Further progress in the field of light localization will depend on the optical purity of the materials. Optical fibres with an extinction length of many kilometres are now quite routine. But unlike optical fibres, which have a refractive index of about 1.5, materials that localize light must have a refractive index of at least 3.0, and at the same time be optically pure. (Unfortunately, a high index usually means operating close to a resonance, and absorption also becomes large near a resonance.) Candidates include silicon and germanium, either powdered or in the ordered form of a photonic band-gap material. These developments bring closer the new age of photonics, in which zero-threshold micro-lasers, sub-picosecond optical switches and all-optical transistors will take over from conventional electronics. □

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Spongiform encephalopathies

B lymphocytes and neuroinvasion

Paul Brown

The latest episode in the long-running serial of scrapie pathogenesis appears on page 687 of this issue¹, with a further molecular dissection of genetically altered mice. The Swiss team¹ has taken advantage of the fact that the gene encoding the prion protein PrP — the leading candidate for the cause of all transmissible spongiform encephalopathies, including scrapie and Creutzfeldt–Jakob disease (CJD) — can be made inoperative (that is, 'null'), without evident harm to the mice². Working backwards from the target organ of disease (the brain) to peripheral (intraocular, intravenous or intraperitoneal) sites of infection, the authors have used surgical transplants in combination with normal and genetically altered mice in an effort to decode the pathogenesis of disease at the molecular level. They show that differentiated B lymphocytes are

important for neuroinvasion — a finding with both public-health and therapeutic implications.

Intracerebral inoculation of the scrapie agent into a null mouse does not produce disease. However, intracerebral inoculation of a null mouse harbouring a small, PrP-expressing neural transplant destroys the graft, although the surrounding tissue is free from disease. In contrast, inoculation by peripheral routes does not destroy the graft: intraocular inoculation fails despite the fact that, in normal animals, the optic nerve is an efficient route of brain infection; and intraperitoneal or intravenous inoculation do not destroy the graft, even after lethal irradiation and transplantation of PrP-expressing haematopoietic stem cells (although these mice do develop infection of the spleen).

This series of experiments builds on a foundation of studies dating back to the 1960s, when Pattison and Millson established the widespread extraneural distribution of infectivity in scrapie-affected sheep and goats. The chief architects during the 1970s were Hadlow and Ecklund, followed, in the 1980s, by Fraser and Dickinson, and by Kimberlin and Walker. The importance of their contributions cannot be overemphasized in an era in which the power of molecular biology and accelerated publication has created a misguided tendency to neglect as already dated any research completed more than a few years earlier.

Thanks to them, and subsequent researchers (Kuroda, Diringer, Kitamoto and Lasmézas), we already know a good deal about the pathogenesis of transmissible spongiform encephalopathies. After introduction of the scrapie agent into various peripheral sites, including the stomach, infectivity first appears in the lymphoreticular system — tonsils, thymus, lymph nodes, and especially in spleen, in which a primary phase of replication occurs in B-cell-rich tissue fractions. Infectivity in these and other widely separated body organs implies that the infectious agent travels through the bloodstream, and this has been explicitly shown by isolation of infectivity from blood during both the pre-clinical and clinical stages of disease. Only after establishment of infectivity in peripheral organs does infectivity (and PrP) become detectable in the central nervous system; first in the spinal cord, and then in the lumbar and cervical cord segments and brain stem. Finally, genetically immunodeficient mice can partially resist intraperitoneal infection, yet they recover almost full susceptibility if immunologically reconstituted by the intraperitoneal injection of normal spleen cells.

This essential schema of scrapie pathogenesis is equally valid whether the infectious agent is a peculiar little virus or (as the Swedish Academy has just authorized) the prion protein. The observation by Klein *et al.*¹, that mature B lymphocytes participate in neuroinvasion, should lead to an increasingly precise appreciation of the molecular basis of scrapie pathogenesis. For example, do B lymphocytes act as blood-borne carriers of the infectious agent, or do they act while in the spleen? Serial-infectivity measurements of blood lymphocytes and spleen during the incubation period could be compared in normal and B-cell-deficient mice. Blood infectivity could also be evaluated in splenectomized B-cell- and T-cell-deficient mice. What would happen after infection of a null mouse with PrP-expressing intracerebral and peripheral nerve implants, or intracerebral and splenic grafts? And could combinations of immunodeficient and PrP null mice strains be created?

Neuroinvasion may turn out to have a hierarchy of options. B lymphocytes contacting terminal nerve endings in the spleen could be the most efficient route; other lymphoid-tissue sites and transfer cells might be less efficient; and direct contact with nerve endings (short-circuiting the lymphoreticular system) least efficient of all. Physical transfer of the infectious agent from a lymphoid cell to a neuron might also require a ligand molecule — the mysterious 'protein X', for example. Among the candidates are two proteins with an affinity for PrP, which have been proposed as cell-membrane PrP receptors in this month's issue of *Nature Medicine*^{3,4}.

What about the public-health and therapeutic implications of a role for B lymphocytes in peripherally acquired disease? There is a convincing link between the 'new variant' of Creutzfeldt-Jakob disease (vCJD) and consumption of infectious tissues from cattle with bovine spongiform encephalopathy (BSE). Moreover, the infectious agents of scrapie and CJD can be isolated from blood or blood components. The involvement of B lymphocytes in neuroinvasiveness adds another reason to consider a step that will deplete white blood cells during the commercial processing of blood, to reduce the possibility of transmitting CJD through blood products.

The outlook for therapeutic benefits is more problematic. Because we have no pre-clinical test for infection, nor any epidemiological clues as to who is at risk of vCJD, preemptive elimination of B lymphocytes is out of the question. PrP has been found in the tonsils of patients with vCJD, but this has so far been investigated only in clinically ill patients. We have no idea when — or even whether — PrP might appear during the incubation phase of disease. Moreover, even if PrP is present during the incubation period, we know that neuroinvasion occurs well before neurological symptoms. We cannot imagine that entire populations would submit to tonsillar biopsies as a screening procedure for vCJD. Thus, for the moment, we had best not push the implications of the involvement of B lymphocytes in neuroinvasion too far from the realm of basic science. When we know precisely how B lymphocytes influence neuroinvasion, it may be possible to nullify their effect without resorting to wholesale elimination strategies. □

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Daedalus

Music with everything

Music used to be a scarce luxury; but these days it is available all the time and everywhere. The children of the electronic age can hear it 24 hours a day. But some folk still agree with George Orwell, who said that the purpose of light music is to prevent you thinking. Those who enjoy their own thoughts often wish to be without it. Daedalus is now attacking the problem.

The most intrusive music is the stuff that company telephone switchboards play to you while they try to make your connection. You are forced to listen to this pestilent noise in order to tell when it stops. And it's no good retaliating by singing a few bars back when you are finally connected. Your hearer will simply be puzzled: he never hears the stuff himself. So Daedalus is devising a circuit that can distinguish between speech and music, so as to kill the latter.

He points out that the power spectrum of music has regular peaks at the intervals of the musical scale. It should be simple to detect this pattern, and use it to drive a muting switch. You could then wait for your call to be connected in blessed silence, but would still hear the recipient's voice when he came through. If he started to sing, however, or to accompany himself on the banjo, you would lose him: the circuit will cut out any sound with that tell-tale musical periodicity in its power spectrum.

The same principle could tame the radio. News and weather buffs could hear them whenever they came on, without being distracted by the noise between. But what music-haters want most of all is a device to kill music heard through the air — as from Muzak speakers and other people's radios. So far, Daedalus has only a partial answer. His Music Saboteur has a microphone, a music-detector and a processor. When it detects music in the ambient sound, it splits the whole incoming signal into several bands. Some are raised a fraction of a tone while others are lowered; some are advanced in time and others retarded. This mixture is then put out through a speaker as a local spoiling signal. The user hears the incoming sound and the spoiler together. Speech is slurred and roughened by this combination; but music is utterly wrecked. Its frequency and time-domain power spectra are smeared out; its 'catchiness', its hold on the attention, is almost completely lost. It becomes mere formless background noise — still there, alas, but far less annoying.

David Jones

Palissy's philosophical pots

The Huguenot Bernard Palissy is known for decorated grottoes and dishes. But he also helped to lead the way towards the use of empirical methods in science and the debunking of mystic medieval metaphysics.

Martin Kemp

Nowadays we think of the so-called 'applied' or 'decorative' arts as just that — combining functional application with decorative ornament — but not as conceptual vehicles for the kind of high seriousness that we associate with the 'fine arts' of painting and sculpture. But this is not a hierarchy that we can safely apply to art in the past. It is salutary to realise that a Hellenistic chalcedony dish in the Medici collection in Florence in 1492 was valued at 10,000 florins, while the most expensive painting (by Fra Angelico) rated only 100.

The famed ceramic dishes by the eccentric Huguenot potter, Bernard Palissy, now survive as curious items in collections of ceramics, but for the artist and his royal patrons in France his creations were of great visual and philosophical moment.

Palissy was named in 1563 as *Inventeur des Rustiques Figulines du Roy* ('King's Inventor of Rustic Ceramics') and gained renown as the maker of elaborate, semi-subterranean grottoes, including one in the area of the Louvre for Queen Caterina de' Medici. After the death of the widowed queen, he was finally imprisoned for his religious beliefs as a reforming Huguenot and met a melancholy end in the Bastille in 1590.

What distinguished Palissy's work as a grotto designer and potter was his extensive use of life casts of animals, brilliantly coloured with fused enamel glazes. Life casting of animals and plants became the expensive speciality of a number of goldsmiths in the sixteenth century, requiring prodigious skill, particularly for soft-bodied creatures.

Palissy virtually scooped the field in ceramics, having driven himself to exhaustion in mastering the huge technical problems of modelling and firing. His repertoire, most especially his marine creatures and amphibians, stands alongside the compelling images in the great contemporary picture books of natural history. But they are more than objects of supreme naturalism.

Palissy was the author of two extraordinary dialogues on the sciences of the Earth, published in 1563 and 1580. In the second of these, his *Admirable Discourse on the Nature of Waters...*, he exploits his artisan's stance as "a man without Latin" to debunk the elaborate metaphysics of the alchemists and abstruse philosophers. He advocates plain, straightforward explanations of age-



Bernard Palissy's Dish with Snake and other Life Casts.

old mysteries, such as the presence of springs on high mountains, and the formation of fossils.

Typically he attributes fossils to a casting process: "Just as all kinds of metals and other fusible materials take on the shape of the hollows or moulds in which they are placed or thrown, and even when thrown into the earth take the shape of the place where the material is thrown or poured, so the materials of all kinds of rocks take the shape of the place where the material has congealed."

Above all, he saw the fiery "womb of the Earth" mirrored in his own infernal kilns: "The kilns in which I bake my work have taught me much concerning the violence of fire; but among other things they have made me know the strength of the elements which generate earthquakes...."

Like a number of his aristocratic and learned contemporaries, he formed a 'cabinet of curiosities', but his was specifically designed to provide empirical proofs of his geological theories: "I have set up a cabinet in which I have placed many admirable and monstrous things which I have drawn from the womb of the Earth, and which give evidence of what I say, and no one will be found who will not be forced to admit them to be true."

Palissy stands as a supreme representative of the way in which the intrusion of practical knowledge and empirical methods into the philosophical mainstream did so much to fire the revolutionary changes in science around 1600. □

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Archaeological frankincense

Frankincense, or *olibanum*, obtained from trees of the genus *Boswellia*¹, is the best known of the aromatic gum resins used throughout the world as incense in religious ceremonies. The earliest archaeological evidence for the burning of incense comes from the Old Kingdom in Ancient Egypt, where spoon-shaped incense burners with long handles have been found. However, no chemical evidence exists of the exact resin used. A wide range of ingredients would have been used by the ancient incense-maker and such materials would have been important traded products². We have chemically characterized frankincense from the archaeological record at the site of the major frontier settlement of Qasr Ibrim, Egyptian Nubia.

The material (from the Egyptian Exploration Society) came from levels associated with the Post-Meroitic phase of occupation of the site. Samples of amorphous material were recovered during sieving of fill from the cellar of a house dating to around AD 400–500 (Structure X-265)³.

We characterized the resins by a combination of gas chromatography and mass spectrometry (GC/MS) using solvent solubilization (2:1 v/v, dichloromethane/propan-2-ol), derivatization and pyrolysis techniques⁴. Solvent-soluble fractions showed the pres-

ence of pentacyclic triterpenoid components present in modern frankincense resin, as determined by mass spectrometry (Fig. 1). Especially characteristic are the α - and β -boswellic acids (1 and 2) and the corresponding O-acetyl derivatives (3 and 4), which dominate the GC/MS mass chromatograms obtained from both the ancient and modern frankincense (Fig. 2).

These data were supported by detection of 24-noroleana-3,12-diene and 24-norursa-3,12-diene, which are the most abundant components of the Curie-point (610 °C) pyrolysates of the extracted archaeological resin, and reference frankincense. The boswellic acids and their O-acetyl derivatives are the major constituents of the fresh aromatic gum resins from *Boswellia* trees¹.

Other pieces of amorphous resin recovered from the same excavations at Qasr Ibrim comprised tricyclic diterpenoid acids including isopimaric acid, abietic acid and dehydroabietic acid, indicating a pinaceous resin⁵. Although such resins and their derivatives have been reported at archaeological sites⁶, we believe that this is the first occasion where diterpenoid and triterpenoid resins have been recovered from the same locality.

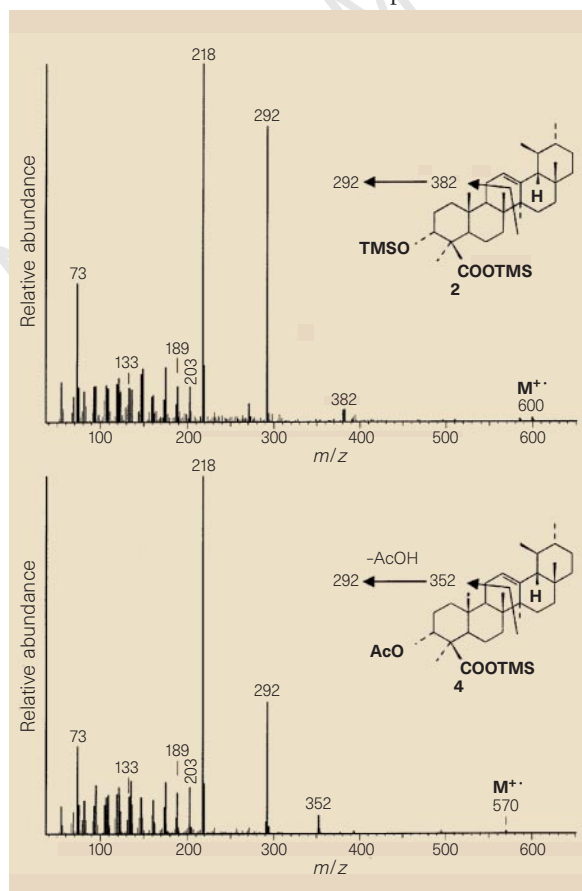
Difficulties in detecting frankincense, and similar commodities, in the archaeo-

logical record stem from their amorphous nature, which means they are easily overlooked in excavations.

Boswellia did not grow near Qasr Ibrim, being found mainly in northern Somalia and southern Arabia⁷. The frankincense must therefore have been imported to the middle Nile. The Ancient Egyptians obtained incense from northern East Africa, specifically from the ill-defined area of Punt, but there is no chemical evidence that the material was true frankincense. The earliest documentary evidence for the use of frankincense is in biblical references in the seventh century BC^{8,9}.

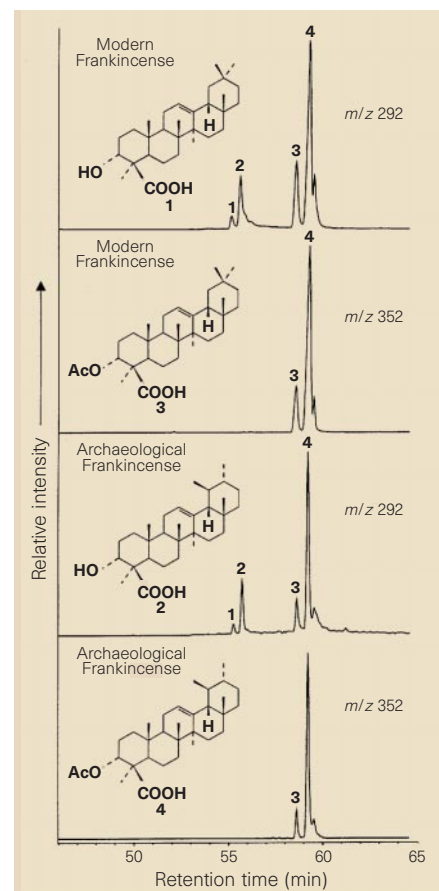
The main period of trade was the Greco-Roman period, when frankincense was shipped by sea from southern Arabia and possibly overland by caravan. Like *Boswellia*, Pinaceae did not grow near Qasr Ibrim, so the pine resin must also have been imported.

The discovery of similar-sized pieces of the two types of resin in close association may point to their use together in incense-burning ceremonies. By the late Roman period most frankincense seems to have been obtained from the African side of the Red Sea, but the arrival of Christianity in the Mediterranean and Egypt apparently reduced demand for it¹⁰. This coincides with the date of our finds of frankincense at



◀ **Figure 1** Mass spectrometry of triterpenoids of ancient frankincense. Electron ionization (70 eV) mass spectra of the trimethylsilyl derivatives of β -boswellic acid (upper) and O-acetyl- β -boswellic acid (lower) obtained from the GC/MS analysis of ancient frankincense. Insets: structures showing the origins of the fragment ions used to plot characteristic mass chromatograms (Fig. 2).

▶ **Figure 2** Comparison of ancient and modern frankincense. Partial GC/MS mass chromatograms of the trimethylsilylated solvent-soluble fractions of a sample of reference frankincense (from R. White) and of resin recovered from Qasr Ibrim. The structures shown are: 1, α -boswellic acid; 2, β -boswellic acid; 3, O-acetyl- α -boswellic acid; 4, O-acetyl- β -boswellic acid. The m/z 352 mass chromatograms characterize O-acetyl derivatives, the major components of both the ancient and modern resin. The m/z 292 chromatograms show the similarity of the distributions of the O-acetyl and free alcohol forms. Inset: structures of compounds in their natural (underivatized) forms.



Qasr Ibrim^{3,11}.

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How pine cones open

The scales of seed-bearing pine cones move in response to changes in relative humidity. The scales gape open when it is dry, releasing the cone's seeds¹. When it is damp, the scales close up. The cells in a mature cone are dead, so the mechanism is passive: the structure of the scale and the walls of the cells composing the scale respond to changing relative humidity. Dissection of cones from the Monterey pine, *Pinus radiata*, revealed to us two types of scale growing from the main body of the cone — the ovuliferous scale and the bract scale. The larger ovuliferous scales respond to changes in relative humidity when removed from the body of the cone.

The scale consists of two tissues distinguishable with the naked eye (Fig. 1a). The inner surface of the scale is composed of sclerenchyma fibres (8–12 μm in diameter, 150–200 μm long), grouped in bundles reminiscent of cables. The outer surface of the scale is composed of sclerids (20–30 μm diameter, 80–120 μm long).

We mounted a scale on a rigid metal frame and exposed it to controlled and

changing relative humidity at 23 °C in an enclosed chamber. Using image analysis, we measured the angle between the scale and the base of the frame, and the distance that the tip of the scale moved. The scale moves towards the centre of the cone in high relative humidity and away from the centre in low relative humidity (Fig. 1b).

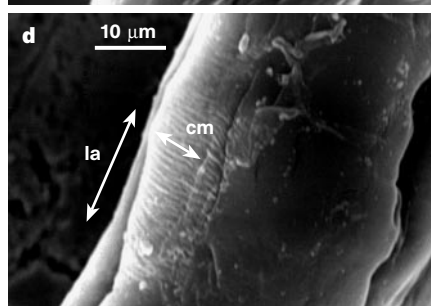
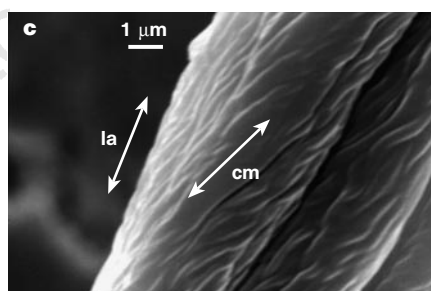
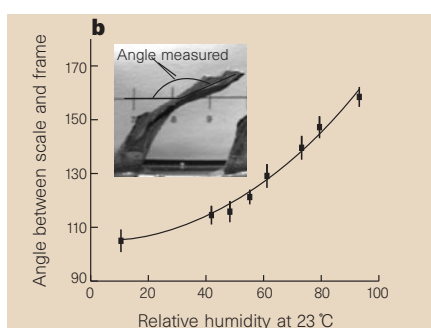
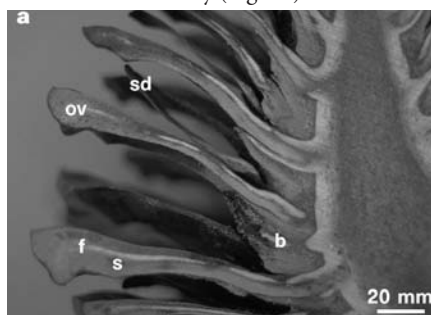


Figure 1 Morphology and behaviour of pine cone scales. **a**, Median longitudinal section of female pine cone. **b**, bract scale; **sd**, seed; **ov**, ovuliferous scale with two-layer structure consisting of **f**, fibres (white line within the scale) and **s**, sclerids. **b**, Graph plotting the angle a scale makes to the base of the experimental apparatus against relative humidity. Inset: experimental apparatus and measured angle. Five scales were used to calculate mean $\pm$ s.e.m. **c,d**, Scanning electron micrographs of fibres and sclerids, respectively. θ , the angle between the long axis (**la**) of the cell and the direction of winding of cellulose fibres (**cm**), is high in sclerids and low in fibres.

We exposed sclerid and fibre cells to a range of relative humidities in a microbalance with a controlled environment and measured the weight changes with time. There were no differences between the two cell types. Chemical analysis² showed that each cell type has roughly a 20% volume fraction of cellulose in its cell wall. The rest is lignin, hemicellulose and pectin.

There are large differences in the tensile stiffness (fibre 4.53 ± 0.90 GPa; sclerid 0.86 ± 0.05 GPa). With a 1% change in relative humidity at 23 °C the coefficient of hygroscopic expansion of fibres (0.06 ± 0.02) is significantly lower than that of sclerids (0.20 ± 0.04). Modelling the scale as a simple bilayer structure requires that three parameters are known³: the stiffness of the two tissue types, the relative dimensions of each layer and their coefficient of hygroscopic expansion. The movement of the tips of the scales is not significantly different from that predicted by the model⁴ (mean, 16.2 mm; predicted, 20.6 mm; $t = 2.25$; 8 d.f.; not significant).

It is not possible to dissect individual cells from the scale as the material is extremely tough. We removed cells using chemical maceration but this removes water and some of the other components of the cell wall. This may affect the observed angle of winding of the microfibrils relative to the long axis of the cell (θ), as may the extremely dry condition under which the cells were observed. Scanning electron micrographs show that θ is considerably lower in fibre cells than in sclerids (Fig. 1c, d). This was confirmed by polarizing light microscopy⁵, which indicated that θ is $30^\circ (\pm 2^\circ)$ for fibre cells and $74^\circ (\pm 5^\circ)$ for sclerid cells.

The mechanism of bending therefore seems to depend on the way that the orientation of cellulose microfibrils controls the hygroscopic expansion of the cells in the two layers. In sclerids, the microfibrils are wound around the cell (high winding angle) allowing it to elongate when damp. Fibres have the microfibrils orientated along the cell (low winding angle) which resists elongation. The ovuliferous scale therefore functions as a bilayer similar to a bimetallic strip, but responding to humidity instead of heat.

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Managing clinical uncertainty

The Progress of Experiment: Science and Therapeutic Reform in the United States, 1900–1990

by Harry M. Marks

Cambridge University Press: 1997. Pp. 258.

£45.00, \$59.95

John Harley Warner

How do we know that a particular drug is effective at the bedside, as well as in the marketplace? What reliable guides do we have for the clinical investigations of an academic medical elite and for the daily prescribing habits of general practitioners? Which intellectual and social tools are most dependable for defining therapeutic merit in theory, practice and law?

The shifting ways in which twentieth-century therapeutic reformers in the United States have grappled with such questions is the focus of Harry M. Marks's bold, nuanced account.

In a century flooded with pharmaceutical marvels (and some notorious iatrogenic tragedies), reformers have seen in experimentalism the most promising foundation for creating a rational therapeutics. Marks is not chiefly concerned with the ways in which new therapeutic possibilities were developed, or with recounting successes and failures. Rather, the story he tells is about how the methods and ideals of science were brought to bear on the management of clinical uncertainty.

The nature and meanings of experimentalism have changed over time, Marks convincingly shows, but its place in the larger programme of therapeutic reformers to bring scientific order to clinical practice remained remarkably durable. Their task was to create reliable criteria for therapeutic evaluation, and to inculcate their conception of a scientific attitude towards therapeutics among the medical rank and file.

Today, the randomized, controlled clinical trial, although not without its critics, is the gold standard of clinical research. Yet even as recently as 1950, therapeutic evaluation relied little on statistics. Early in this century, progressive therapeutic reformers distrustful of commercialism looked on the integrity of experienced researchers consecrated to the ideals of experimental science as the most dependable haven for clinical practice. In 1906 the American Medical Association founded its Council on Pharmacy and Chemistry to judge independently the claims made by drug companies for their products.

Reformers went on to promote cooperative investigations as a means of pooling expert experience, but it was the character of individual clinicians that was believed to best guarantee the integrity of their therapeutic



Jab now, no pain later: a doctor gives typhoid inoculation at a rural school in Texas in the 1940s.

observations. During the 1930s and 1940s, officials at the US Food and Drug Administration largely adopted the council's approaches to therapeutic assessment in their efforts to regulate drug safety.

After the Second World War, however, therapeutic reformers increasingly sought to purge clinical evaluation of subjectivity, and began to embrace the double-blind, randomized, controlled clinical trial as a more impersonal, scientific standard for assessing and improving therapeutic knowledge and practice. Building on the statistician R. A. Fisher's conception of experimental design, experiments by the British Medical Research Council and the US Public Health Service to use streptomycin to treat tuberculosis during the war introduced the randomized clinical trial.

From the 1950s onwards, Marks observes, therapeutic reformers insisted that trust in numbers — in an experimental method regulated by statistics — should supplant the trust an earlier era had placed in the judgement of experienced researchers. Investigations at the bedside, newly governed by the reign of statistics, could be every bit as scientific as research conducted in the experimental laboratory.

Marks anchors his overarching account of change over time in a series of richly textured case studies, offered, as he rightly insists, not as typical but as powerful exemplars of larger patterns. Prominent nodal points include, from the period between the two world wars, the Cooperative Clinical Group's study of syphilis treatments and the Commonwealth Fund's experiments with serum treatment of pneumonia; the National Research Council's

investigations of penicillin during the Second World War and the streptomycin studies conducted by the Veterans Administration and the Public Health Service immediately after the war; and, in the 1960s, the Diet-Heart study and the University Group Diabetes Program study of tolbutamide.

It is through these case studies that we are drawn into the world of therapeutic investigation, including conflicts between the aspirations of researchers and the day-to-day demands on general practitioners; between the purity of experimental design and the realities of patient compliance; and — in the book's most contemporary example, which is invoked but not thoroughly explored — between the political and clinical demands of patient activists and the protocols of therapeutic research on HIV and AIDS.

Readers might well feel uneasy, as I did initially, about Marks's use of the omnibus category "therapeutic reformers", which encompasses a motley assemblage of pharmacologists, physiologists, statisticians, epidemiologists, journal editors, government physicians and clinical specialists. I would also have liked to learn more about how the American medical profession at large perceived the movement for therapeutic reform and its products; the strategies enlisted to persuade general practitioners of the virtues of randomized, controlled clinical trials; and how what Marks calls a "quintessentially an American story" compares with patterns in other national contexts.

What more than warrants his use of the aggregate term "therapeutic reformers",

however, is the way it underscores how the leading characters in this study constituted a community of believers, a diverse and changing group of physicians and scientists tightly bound together over time and across disciplinary boundaries by their pervading faith that an unfaltering commitment to science would overcome all social, technical and political obstacles to a rational therapeutics. This approach also underscores the usually unacknowledged political nature of this community, and helps to explain what Marks identifies as the problematic conviction of therapeutic reformers that their allegiance to science should make them alone the arbiters of clinical trials and clinical care.

No breezy narrative, this is a challenging historical study, sophisticated in its research and justifiably confident in its analysis. Marks has given us the best study we have of the shifting place and meanings of science in the culture of twentieth-century American clinical medicine. And as the field of medical history increasingly turns its attention to the twentieth century, this work is both a model and a springboard for future scholarship. No one who wishes to understand the world of therapeutic investigation, regulation and practice that doctors and patients live with today can afford to miss this important and engrossing study. □

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Molecular thriller

Principal Investigation

by B. B. Jordan

Berkley: 1997. Pp. 305. \$5.99 (pbk)

Maxine Clarke

Merging scientific with criminal detection, the pseudonymous B. B. Jordan provides an entertaining couple of hours in which to learn the basics about infection and allergy. As a crime thriller, *Principal Investigation* holds its own, at times precariously close to, but just about avoiding, standard genre clichés of mad scientists, WIJ (woman in jeopardy) and 'Nam flashback syndrome.

The energy of the main character, lab chief Celeste Braun (equal parts PC and PI), is daunting: travelling to Japan to become security consultant to a global biotechnology corporation, writing a grant proposal, supporting an ex-colleague through accusations of scientific fraud and a congressional investigation, jogging round the park, giving three lectures in three different places, constructively combating Japanese sex discrimination, flirting with a man at the airport — all within the first 13 pages of her appearance.

The exhausting pace doesn't let up for the rest of the book. Although this makes for a racy read, too much is piled in and left hanging: US–Japan relations; an attack on the

(slightly disguised) Office of Scientific Integrity; a Watergate-style investigation; the ethics of the military's vaccine trial programme; the financial struggles of the publicly funded medical school of the University of California at San Francisco (again, thinly disguised) compared with commercial research; an assessment of the peer-review system; and so on.

Far too many coincidences are invoked to weave in all these plot strands: ex-colleagues crop up as TV interviewees, air-crew, journalists, in queues at foreign libraries, or walking in the street, naturally always at crucial times to keep the plot moving.

Jordan would have done better to have limited the scope of the book to the (complicated enough) story about a rogue scientist's evil plans, woven in with the explanations of pollen allergy, HPLC and so on. The added complications, together with the non-existent characterization, result in a confusing whole; indeed, some subplots are logically inconsistent. (And rather too much depends on an otherwise supercharged heroine not having e-mail or checking her phone messages.)

This is a pity, because the factors leading to the dénouement are neatly done, depending on both the basic biology explained earlier in the book and the wits of the protagonist(s). Basic immunology could never be so engagingly described in a textbook, or be so digestible. □



Turning back the tide of destruction in the world's forests

Commercial logging operations and clearance for agriculture are wreaking havoc in forests around the world, as conservationists frequently remind us. So it is refreshing to come across a book that presents positive stories of forest conservation. *Forests of Hope: Stories of Regeneration* by Christian

Küchli (Earthscan/New Society, £19.99, \$29.95, pbk) describes communities caring for trees. We read, for example, of the residents of Hu Zhai, China, who are planting trees in a battle against erosion; and of the women of Kitui in Kenya whose tree planting is crucial to the community's livelihood.

Localization of light in a disordered medium

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Among the unusual transport properties predicted for disordered materials is the Anderson localization¹ phenomenon. This is a disorder-induced phase transition in the electron-transport behaviour from the classical diffusion regime, in which the well-known Ohm's law holds, to a localized state in which the material behaves as an insulator. The effect finds its origin in the interference of electrons that have undergone multiple scattering by defects in the solid²⁻¹⁰. A similar phenomenon is anticipated for multiple scattering of electromagnetic waves, but with one important simplification: unlike electrons, photons do not interact with one another. This makes transport of photons in disordered materials an ideal model system in which to study Anderson localization¹⁰⁻¹⁷. Here we report direct experimental evidence for Anderson localization of light in optical experiments performed on very strongly scattering semiconductor powders.

Multiple scattering of light is a common phenomenon in daily life, occurring for example in sugar, fog, white paint and clouds. The propagation of light in these media can in general be described

by a normal diffusion process. For diffusion of light through a disordered material the same Ohm's law holds as for diffusion of electrons through any common resistor: the transmission, or conductance, decreases linearly with the system length (thickness).

Anderson localization brings classical diffusion to a complete halt. That is, on increasing the amount of scattering beyond a critical value, the material makes a transition into a localized state (see Fig. 1). This transition can best be observed in the transmission properties of the system. In the localized state, the transmission coefficient decreases exponentially instead of linearly with the thickness of a sample. At the transition, the transmission coefficient is expected to have a power-law dependence on the inverse thickness, which is probably quadratic^{12,18}.

The main difficulty in the search for localization of light has been the realization of strong enough scattering. The appropriate measure for the amount of scattering is the mean free path l for the light in the medium, times the magnitude of the wavevector k . Localization is expected for $kl \leq 1$ (ref. 19), which is known as the (modified) Ioffe-Regel criterion. This criterion can be understood intuitively if one realizes that below $kl = 1$, the electric field can not even perform one oscillation before the wave is scattered again. So far, localization effects have been reported only for microwaves in a two-dimensional system of rods²⁰ and for microwaves in a confined geometry (a copper tube filled with metallic and dielectric spheres)^{21,22}. In the latter experiment the absorption was very large, which makes the interpretation of the data complicated. The disadvantage of experiments with microwaves compared to light waves is that it is difficult to avoid absorption.

We have been able to realize very strongly scattering samples for light waves, using semiconductor powders. Semiconductors can have a very large refractive index, while, for wavelengths in the

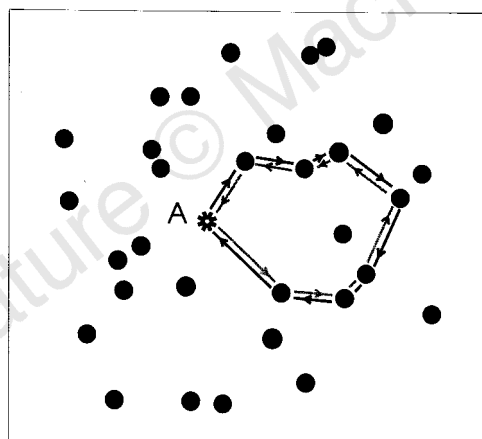


Figure 1 Anderson localization of waves in disordered systems originates from interference in multiple elastic scattering. Here we consider a light source (like an excited atom emitting a photon) in a disordered medium at position A. The light source is denoted by a star symbol and the spheres denote the scattering elements. A random light path that returns to the light source can be followed in two opposite directions. The two waves which propagate in opposite directions along this loop will acquire the same phase and therefore interfere constructively in A. This leads to a higher probability of the wave coming back to A and consequently a lower probability of propagating away from A. On decreasing the mean free path l , the probability for such looped paths increases and at strong enough scattering the system makes a phase transition from the normal conducting state into a localized state, due to interference. In the localized regime, the system behaves as a non-absorbing insulator. Light which is incident on, for example, a slab would be almost completely reflected and the remaining transmission would decrease exponentially with the slab thickness.

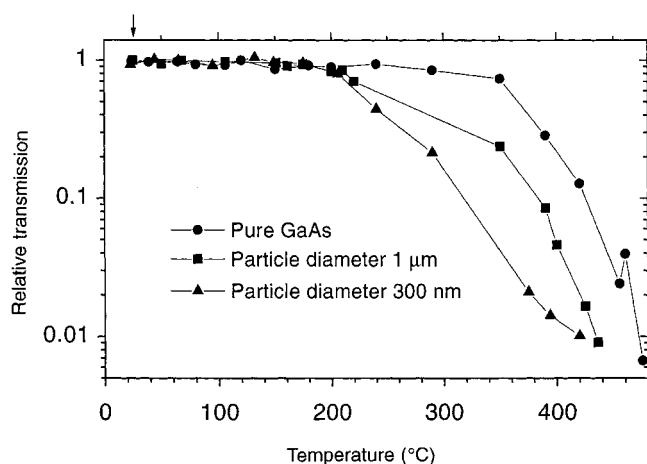


Figure 2 Comparison of the temperature dependence of the transmission of pure GaAs crystals and powders. Data obtained using particles of size of 1 μm and 300 nm are shown. The transmission of the powder samples is measured at a thickness where the total transmission equals 1% at room temperature. All curves are scaled to have a maximum of 1. On increasing the temperature T , the bandgap shifts to lower energies $E_g(T)$ corresponding to higher wavelengths $\lambda_g = 1.24/E_g$ (in μm). The temperature dependence is given by the empirical relation: $E_g(T) = E_g(0) - \alpha T^2/(T + \beta)$, where (for GaAs) $E_g(0) = 1.522$, $\alpha = 8.871 \times 10^{-4}$ and $\beta = 572$, with T in K. This temperature-dependent shift enables the scanning of the region of the bandgap around the laser wavelength, without changing the laser wavelength itself. For example, a temperature of 200 °C corresponds to a wavelength shift of 65 nm. The arrow at top left shows the temperature where we performed all other experiments. We note that the band edge becomes less steep upon grinding the GaAs crystal; this is probably due to the increased importance of surface states and, for example, lattice deformations. But the region close to the laser wavelength remains unabsorbing.

bandgap, the absorption is extremely small. We used light at wavelength $\lambda = 1,064$ nm, at which the absorption coefficient κ of pure GaAs is $\kappa \ll 1 \text{ cm}^{-1}$ and the refractive index is 3.48. Our samples consist of pure (99.999%) gallium arsenide (GaAs) crystals which were ground (as a suspension in methanol) by hand in a ceramic mortar and in a planetary micromill at low speed. By varying the grinding time, we obtained samples with different average particle sizes and thereby different amounts of scattering. In grinding semiconductors one has to be careful not to introduce absorption at the wavelength at which the experiments are performed. For a powder, surface states could become more important and the grinding process (even if performed with little force) could introduce strain or lattice deformations. Surface states, strain and lattice deformations can lead to absorption tails at the edge of the bandgap. We have characterized the change of the band edge of our material by measuring the temperature dependence of the transmission (Fig. 2).

We observe (Fig. 2) that the band edge indeed becomes less steep on grinding, and that the onset of absorption shifts into the bandgap. It is also clear, however, that the region of the absorption tails is still far enough (65 nm) below our laser wavelength as to ensure that these tails will not introduce absorption in our experiments.

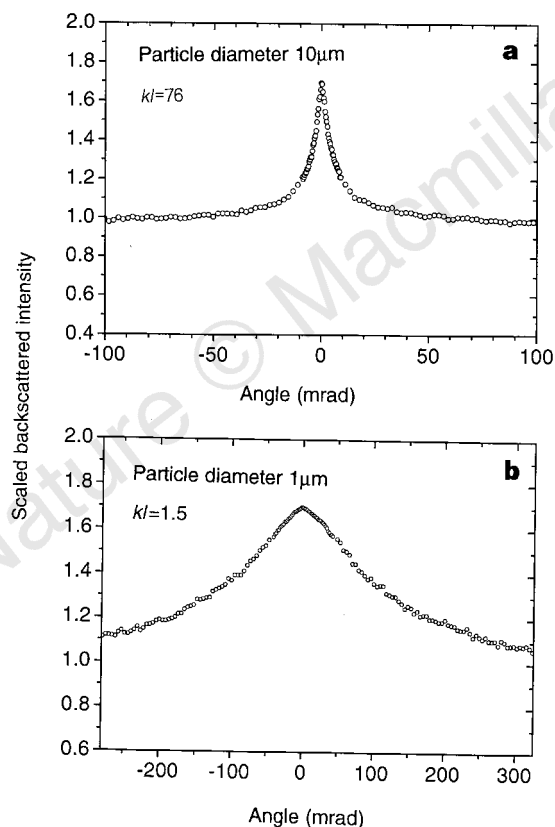


Figure 3 Coherent backscattering cones from coarse-grained (a) and fine-grained (b) GaAs powder. The samples are strongly polydisperse with an average particle diameter of 10 μm and 1 μm, respectively. The sample is illuminated with a mode-locked Nd:YAG laser operating at 76 MHz, with a wavelength of 1,064 nm, pulse duration 100 ps, beam diameter 6 mm and incident power 100 mW. In all experiments we have checked that nonlinear absorption processes like two-photon absorption do not play a role; this was done by lowering the incident power to 20 mW, and finding that this reduction did not influence the results. Detection is performed in the polarization-conserving channel. The mean free path is calculated from the angle of the cusp²⁷, taking into account internal reflection²⁹. This yields $l = 8.5 \mu\text{m}$ and $kl = 76$ for the data in **a**, and $l = 0.17 \mu\text{m}$ resulting in $kl \approx 1.5$ for the data in **b**.

To characterize the mean free path l of our samples, we used coherent backscattering. This phenomenon is a general interference effect between counter-propagating waves, which leads to a narrow cone in exact backscattering²³. It is seen as the precursor to Anderson localization and is therefore also called 'weak localization of light'^{24,25}. The width of the backscattering cone and the angle of its cusp are inversely proportional to l (refs 26, 27) and therefore enable the determination of the amount of scattering inside the samples. Furthermore, the cusp is due to a summation up to (in principle) infinite path length, and is therefore only present in the absence of absorption^{26–28}. In Figure 3, we show backscattering cones for a coarse-grained (Fig. 3a) and a fine-grained (Fig. 3b) powder with an average particle diameter of 10 μm and 1 μm, respectively. From the angle of the cusp we find for the coarse-grained powder $l = 8.5 \mu\text{m}$ and hence $kl = 76$. For the fine-grained powder we find $l \approx 0.17 \mu\text{m}$ which corresponds to $kl \approx 1.5$. The observed value of $kl = 1.5$ is four times smaller than the smallest kl values that have been reported so far³⁰ for light waves. The shape of the coherent backscattering cone at the localization transition is interesting from both an experimental and a theoretical point of view, but will be discussed elsewhere.

To observe a possible localization transition, we have measured the transmission coefficient as a function of sample thickness. In

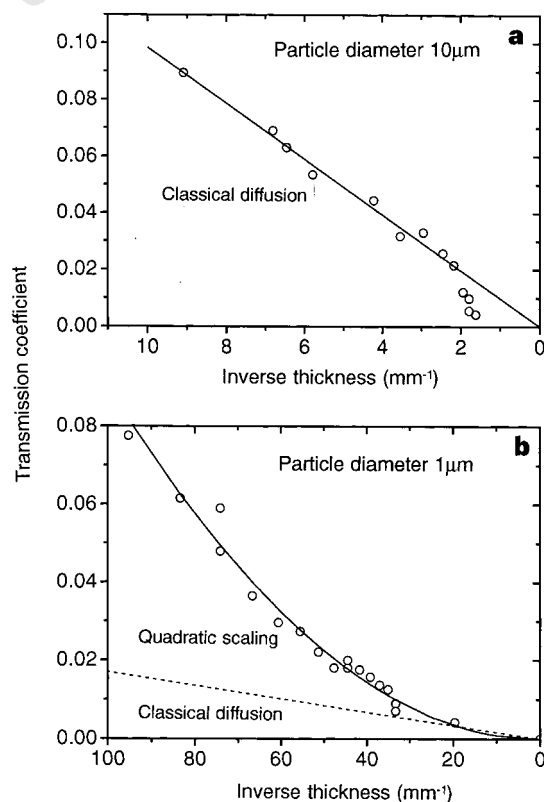


Figure 4 Comparison of the transmission coefficients for two GaAs powders with different average particle diameters. Here the transmission coefficient is defined as the ratio between total transmitted flux and the incident flux. The total transmission is measured with an integrating sphere placed in contact with the back of the sample. The light source is as in Fig. 3, with beam diameter 0.5 mm and incident power 20 μW. The data in **a** correspond to particle diameter of 10 μm and behave completely classically. The solid line is $T = l/L$, with $l = 9.8 \mu\text{m}$, which is in agreement with the backscattering data. The data in **b** correspond to a particle diameter of $\sim 1 \mu\text{m}$. The dashed line is the theoretical curve for classical diffusion with $l = 0.17 \mu\text{m}$, as obtained from the backscattering data. The solid line is a quadratic fit to the data. This quadratic dependence of the transmission coefficient on the inverse thickness is the expected behaviour at an Anderson localization transition.

Fig. 4a, the transmission coefficient is shown for the coarse-grained powder. We see that the transmission behaves completely classically with $l = 9.8 \mu\text{m}$, which is consistent with the coherent backscattering data. At very large sample thicknesses ($L > 500 \mu\text{m}$), the transmission decreased more rapidly than linear due to the onset of absorption. This gives an upper limit for κ (defined as $\kappa = l_m^{-1}$) for the coarse-grained powder, of $\sim 0.13 \text{ cm}^{-1}$, which shows again that the grinding process did not introduce any absorption.

In Fig. 4b, the transmission coefficient T is shown for the fine-grained powder. The dashed line is the theoretical curve for classical diffusion assuming $l = 0.17 \mu\text{m}$ as obtained from the backscattering data. Whereas for classical diffusion T would decrease linearly with the sample thickness L , we find for these samples a quadratic dependence ($T \propto L^{-2}$). This is exactly the behaviour predicted by the scaling theory of localization at the localization transition^{12,18}. We note that no classical diffusion process can show a quadratic system-size dependence.

If we decrease the particle size even further we expect (from Mie-scattering theory) to obtain even stronger scattering. In Fig. 5, the transmission coefficient is shown for a powder with an average particle diameter of 300 nm. In Fig. 5b we see that the quadratic behaviour has changed into an exponential decay in this case, as expected in the localized regime. The transport of light has come to

a halt owing to interference, and the system has made a phase transition from a conducting into a localized state. The characteristic length scale for the exponential decay is called the localization length, which here is $4.3 \mu\text{m}$.

Different techniques could be used to map out the complete localization transition. The amount of scattering could be varied by changing the average particle diameter, the refractive index contrast, the particle density and the wavelength of the light. An important experiment would be a time-resolved transmission experiment in which the reduction of the diffusion constant at the Anderson localization transition is observed. Localization of classical waves is, in many ways, similar to Anderson localization of electrons. There are, however, also interesting differences between light and electrons. For experiments with light waves, coherent (laser) sources are available; the wavelength of such sources may be easily adjusted. Furthermore, for electromagnetic waves photon-photon interactions can be neglected, whereas in the case of electrons, electron-electron interactions always play a role. This latter property in particular makes light in strongly disordered media an interesting system in which to study the Anderson localization transition. $\square$

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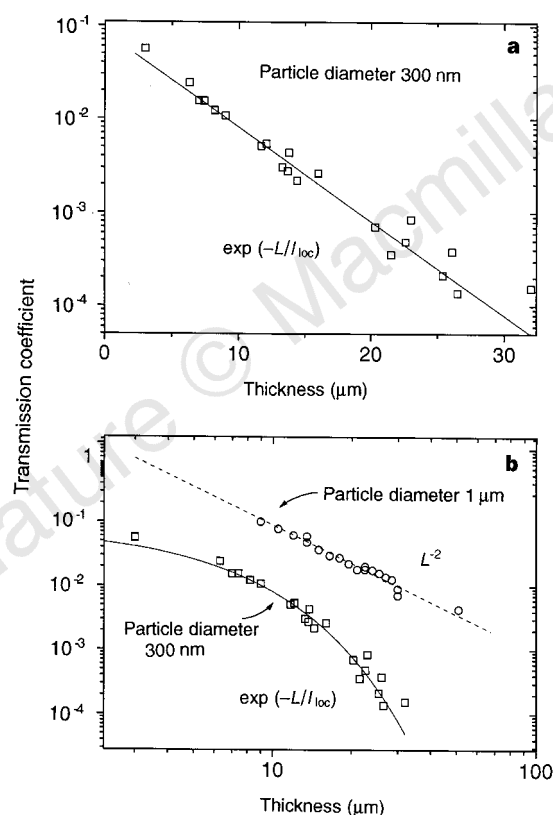


Figure 5 The transmission coefficient of very fine GaAs powder as a function of thickness. The average particle diameter is 300 nm. In **a**, the data are plotted on a semi-logarithmic scale. The solid line is an exponential fit $\exp(-L/l_{\text{loc}})$ with a localization length of $l_{\text{loc}} = 4.3 \mu\text{m}$. In **b**, the same data are plotted on a double-logarithmic scale and the data of Fig. 4b are imported for comparison. We see that for the very fine powder, the quadratic behaviour changes to an exponential decay. The system goes into a localized state where it behaves as a (non-absorbing) insulator.

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Microscopic patterning of orientated mesoscopic silica through guided growth

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The supramolecular assembly of surfactant molecules at a solid-liquid interface can produce tubular structures with diameters of around 10 nm (refs 1–4), which can be used for the templated polymerization of mesoporous silica thin films^{3–5}. The orientation of the tubules depends primarily on the nature of the substrate-surfactant interaction. These nanostructured films hold much promise for applications such as their use as orientated nanowires⁶, sensor/actuator arrays^{7–9} and optoelectronic devices¹⁰. But a method of patterning the tubules and orientating them into designed arrangements is required for many of these possibilities to be realized. Here we describe a method that allows the direction of growth of these tubules to be guided by infiltrating a reaction fluid into the microcapillaries of a mould in contact with a substrate¹¹. An electric field applied tangentially to the surface within the capillaries induces electro-osmotic flow, and also enhances the rates of silica polymerization around the tubules by localized Joule heating. After removal of the mould, patterned bundles of orientated nanotubules remain on the surface. This method permits the formation of orientated mesoporous channels on a non-conducting substrate with an arbitrary microscopic pattern.

The development of low-cost lithographic techniques to pattern materials with structural features on the nanometre scale is important for the miniaturization of electronic, optoelectronic and magnetic devices. Existing approaches include technologies involving scanning electron beams^{12,13}, X-ray lithography^{14,15}, scanning probe microscopes^{16,17} and imprinting methods¹⁸. The 'micro-moulding in capillaries' (MIMIC) technique¹¹ provides a low-cost approach to thin-film patterning. In this method, a network of patterned capillaries is formed by placing an elastomeric stamp (typically made of polydimethyl siloxane, PDMS) possessing designed relief features on its surface in contact with a flat substrate (see Fig. 1). Second, an acidified aqueous reacting solution of tetraethoxysilane (TEOS) and cetyltrimethylammonium chloride (CTAC) is placed in contact with the edge of the mould and is transferred by 'wicking' into the networked channels. Typical molar ratios for the reacting solution are 1 TEOS:1.2 CTAC:9.2 HCl:1,000 H₂O. As has been shown previously⁴, the formation of a mesoscopic silica film begins to occur immediately on contact of this solution with any interface onto which the surfactant can adsorb. A dilute solution of the TEOS silica source is specifically used to prevent homogeneous nucleation of inorganic material in bulk solution, and to promote heterogeneous nucleation and growth of a mesoscopic film at the substrate-solution interface⁴.

Within the capillaries, because the reacting solution is dilute, reactants are quickly depleted, and film growth ceases if wicking occurs only through capillary suction. Moreover, the growth of mesoscopic film at the edges of the mould seals the capillaries and prevents diffusion of reacting species to the interior of the mould.

Therefore, to maintain a uniform concentration of reactants within the capillaries during the growth process, we applied an electric field parallel to the substrate in the manner shown in Fig. 1. Application of an electric field in this geometry has three effects: it induces electro-osmotic fluid flow; it aligns surfactant tubules; and it causes localized Joule heating of the solution. These effects are synergistic in guiding and polymerizing the silicate mesostructures within the microcapillary reaction chambers. For applied fields $>0.1 \text{ kV mm}^{-1}$, electro-osmotic fluid flow is observed within the capillaries, as a result of the interaction of the field with the ionic double-layer charge near the capillary wall¹⁹. In our case, surface charge on the capillary walls arises from adsorption of the positively charged CTAC surfactant²⁰. Maintaining a steady fluid flow through the capillaries during the entire growth process ensures that the reactant concentration within each tiny reaction chamber remains constant with time. This constancy is essential for the process and allows uniform films to be grown.

Figure 2 shows scanning electron microscope (SEM) images of lined and two-dimensional patterns of mesoscopic silica grown on a silica substrate after 5 h of reaction time. A d.c. field of 0.15 kV mm^{-1} was applied during the entire reaction process, and fresh reacting fluid was continuously dripped on one side of the mould to replenish the volume removed by the electro-osmotic flow. In each case, the patterns formed replicate the structures of the mould. Within the capillaries, films begin to grow on all exposed surfaces, that is, at both the PDMS mould and the substrate/aqueous-solution interface. As the reaction progresses, the capillaries narrow in the centre and eventually seal completely. The high conductivity of the acidic reaction solution gives rise to significant Joule heating at these applied voltages and accelerates the polymerization rate of the TEOS precursor to silica. The dependence of silica polymerization rates on temperature is a well studied phenomenon²¹. Positioning the electrodes in an excess reservoir of reacting solution outside the microcapillary volume (Fig. 1) allows high fields to be applied across the aqueous solution confined within the capillaries. In such a scheme, rapid electrolysis ($\text{H}_2\text{O} \rightarrow \text{H}_2 + 1/2\text{O}_2$) ensues, but bubble formation is confined to the fluid reservoir near each electrode and does not disturb the formation of silica mesostructures within the capillaries. With no applied field, $0.5\text{-}\mu\text{m}$ -thick films are typically grown in a period of 24 h but the lines show depressions or discontinuities due to incomplete filling; with an applied field of 0.1 kV mm^{-1} , similar

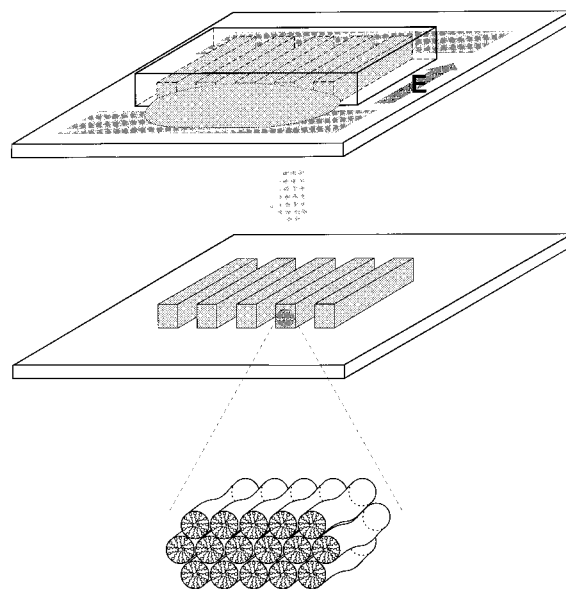


Figure 1 Schematic illustration of the technique used to induce guided growth of mesoscopic silicate structures. The cavity dimensions are not to scale.

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thicknesses are achieved in 1–5 h. Localized heating of the reacting solution in this manner thus provides a useful method of rapidly rigidifying the aligned surfactant tubular structures formed within the microcapillaries.

To determine the orientation of the surfactant nanotubules within these structures, cross-sectional samples were prepared using a Leica ultramicrotome and analysed by transmission electron microscopy (TEM). Figure 3 shows a typical example of the resulting TEM images as well as a typical selected-area electron diffraction (SAED) spot pattern. These reveal a hexagonally packed arrangement of tubules with a nearest-neighbour spacing of 5 nm. Detailed examination of diffraction pattern reveals a slightly distorted packing arrangement, with a deviation of 4% from perfect hexagonal. This distortion may be a result of the accelerated polymerization process described above: with no applied field, no distortion is observed.

Multiple cross-sections were taken of the 1- μm line structures shown in Fig. 2; all these cross-sections appeared identical to the image shown in Fig. 3. This indicates that all tubules within the capillaries are aligned parallel to the substrate, the long axis of the capillary, and the direction of the applied field. The uncalcined samples were very sensitive to the electron beam even at 77 K. Under well-controlled conditions of operation (electron current density at the sample was kept to a low level, $\sim 1 \text{ A cm}^{-2}$, at 120 keV), the observed hexagonal structure lasts for a few seconds. (The required exposure time is about 2–3 s for the bright-field image and 30–50 s for the SAED pattern.) During this period, most of the regions were unavoidably converted to an amorphous-like structure. After such exposure, the diffused and weak ring-like diffraction pattern observed from a 1- μm^2 area is probably due to the electron beam-induced amorphous-like structure, rather than resulting from a real amorphous structure.

The long-range alignment of the tubules has also been confirmed by X-ray diffraction (XRD) methods (L. Zhou *et al.*, unpublished

results). The XRD studies indicated the presence in the uncalcined samples of a minority lamellar phase ($<10\%$) with a lattice spacing of 2.9 nm. The presence of this phase does not alter our claims of the orientation and the continuity of the nanotubules. In the uncalcined samples, orientated and hexagonally packed nanotubules are highly aligned along the surface-normal direction with a mosaic width of $<1.7^\circ$. This observation strongly suggests that the films are continuous as any discontinuity or twist in the hexagonally packed bundles of nanotubules would have resulted in a much larger mosaic width. The nanotubules are less aligned in the plane parallel to the silicon surface, having a mosaic width of 20° . Preliminary experiments on samples calcined at 400°C show that the hexagonal phase is retained even after the samples have shrunk by 25%. (Previous work^{3,4,22} has shown that surfactants can be removed from mesoporous thin films without degrading the structure significantly.)

The long-range alignment is in dramatic contrast to the 'unconfined' mesoscopic silica film synthesis⁴, which always results in a disordered, non-aligned arrangement of tubules on amorphous substrates. In our case, rather than taking on a random configuration, growing tubules are guided within the confined space of the capillary and remain parallel to the walls. This orientation occurs either as a result of the action of the external field (that is, alignment of tubules resulting from polarization body forces that operate in regions of dielectric-constant gradient ($\sim \nabla \epsilon E^2$, where ϵ is the local dielectric constant and E is the local electric field; refs 19, 23, 24)) or by virtue of the confined space within which the reaction is performed. In both cases, the tubules would be aligned parallel to the capillary walls. For field-induced alignment, such configurations minimize the overall electrostatic energy—provided that a difference in dielectric constant exists between the inner and outer volume of the tubule. It is also known that the formation of end-caps in self-assembled surfactant cylinders is not favoured, given their high free energy of formation²⁵. Thus, within a highly confined region, surfactant cylinders will take on configurations which

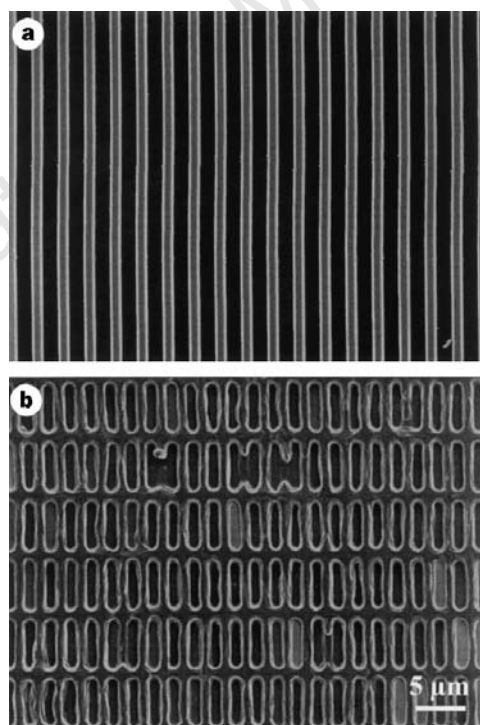


Figure 2 SEM images of 1- μm line (**a**) and two-dimensional (**b**) mesoscopic silicate patterns formed by guided growth within microcapillaries. Electro-osmotic flow is used to transport reacting fluid through the capillaries, and localized Joule heating triggers rapid polymerization of silica around aligned surfactant tubules.

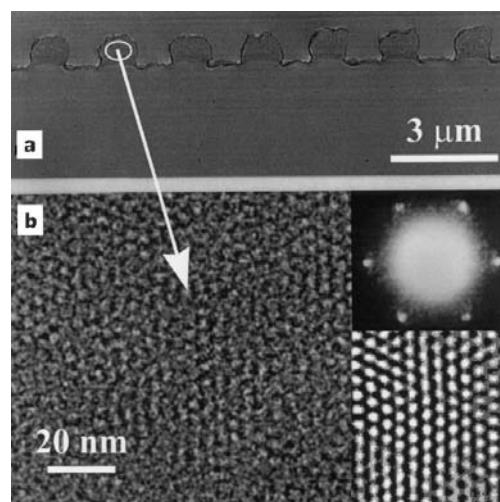


Figure 3 **a** and **b**, TEM images of a patterned mesoscopic silica structure grown on a Thermanox plastic substrate (Electron Microscopy Sciences, Fort Washington, PA). These display a hexagonally packed surfactant tubule structure within the 1- μm -sized lines shown in Fig. 2. The cross-sectional view of each line reveals an identical hexagonally packed pattern of tubules, suggesting global alignment of tubules parallel to the substrate and capillary walls. Similar images have also been obtained for 'confined' films grown on silica substrates. The inset shows (top) the corresponding electron-diffraction pattern obtained from **b**, as well as (bottom) a Fourier transform filtered image. (The same pattern is seen for the full $1 \mu\text{m}^2$ region in **a**.) The diffraction pattern reveals a slightly distorted hexagonal lattice with 4% strain. The strain seems to be caused by the rapid polymerization of the inorganic phase induced by localized Joule heating of the confined reacting fluid.

minimize the number of end-caps. Consequently, they will tend to elongate along the long axis of the capillary rather than truncating at capillary walls.

In the absence of an ordering field, a wide range of slowly curving configurations of tubules is routinely observed⁴. In our case, the combined influence of confining geometry and applied field allows the synthesis of silicate nanostructures with precisely controlled geometries. In this way, the tubule geometry is controlled in all regions of the film and the synthesis can be performed on any required substrate, regardless of the nature of the surfactant–substrate interaction. An enormous variety of patterns can be formed using the MIMIC approach, with nanotubules aligned parallel to capillary walls. Capillary thicknesses of 1 µm, corresponding to ~300 nanotubules, are easily achieved by this method and thinner structures can also be formed using moulds formed from masters prepared by electron beam lithography¹¹.

As a viable method for the production of thin films with complex nanometre- and micrometre-scaled hierarchical architectures, the guided growth of mesoscopic silicates within confined geometries provides a convenient and economic method for fabrication of patterned nanostructured materials. Such materials may be useful in a variety of applications, ranging from sensors and actuators to optoelectronic devices.

Note added in proof: In a recent publication²⁶, a method has been reported for patterning mesoscopic silica through co-assembly of alkanethiol-decorated gold surfaces. However, nanotubules in these films did not show the orientational order observed in our films. □

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A resonance in the Earth's obliquity and precession over the past 20 Myr driven by mantle convection

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The motion of the Solar System is chaotic to the extent that the precise positions of the planets are predictable for a period of only about 20 Myr (ref. 1). The Earth's precession, obliquity and insolation parameters over this time period^{1–6} can be influenced by secular variations in the dynamic ellipticity of the planet which are driven by long-term geophysical processes, such as post-glacial rebound^{5,7–10}. Here we investigate the influence of mantle convection on these parameters. We use viscous flow theory to compute time series of the Earth's dynamic ellipticity for the past 20 Myr and then apply these perturbations to the nominal many-body orbital solution of Laskar *et al.*⁵. We find that the convection-induced change in the Earth's flattening perturbs the main frequency of the Earth's precession into the resonance associated with a secular term in the orbits of Jupiter and Saturn⁵, and thus significantly influences the Earth's obliquity. We also conclude that updated time series of high-latitude summer solar insolation diverge from the nominal solution for periods greater than the past ~5 Myr. Our results have implications both for obtaining precise solutions for precession and obliquity and for procedures that adopt astronomical calibrations to date sedimentary cycles and climatic proxy records.

In many-body orbital calculations, the Earth has traditionally been treated as a non-deformable body^{1,2} with the possible exception of changes in shape associated with tidal dissipation⁴. Recently, some studies have adopted viscoelastic models to investigate perturbations in the Earth's dynamic ellipticity due to surface mass load variations associated with the late Pleistocene ice-age cycles^{8,9}. These studies were motivated by Laskar *et al.*⁵, who suggested that the effect of the ice ages might be sufficient to perturb the Earth's precession and obliquity into a resonance due to the $s_6 - g_6 + g_5$ forcing associated with orbital elements of Jupiter and Saturn. Although this is now believed to be unlikely^{8,9}, the ice-age effect will nevertheless influence astronomical calibrations of Quaternary geological core-records¹⁰. Over periods comparable to the span of current orbital integrations (~20 Myr), secular variations in the dynamic ellipticity of the Earth may arise from a number of geophysical processes, but will be dominated by convective flow in a viscous mantle. The influence of this process on the precession and obliquity of the Earth has not been previously considered.

The dynamic ellipticity of the Earth is defined as

$$H(t) = \frac{C(t) - \frac{1}{2}[A(t) + B(t)]}{C(t)} \quad (1)$$

where C is the polar moment of inertia, A and B are the principal equatorial moments of inertia and t is the time. In the case of a compressible, self-gravitating, newtonian viscous mantle, perturbations in the ellipticity can be computed using the equation¹¹

$$\delta H(t) = -\frac{4\pi a^4}{\sqrt{5}C} \int_{r_{\text{cmb}}}^a G_2 \left[\nu(r) / \nu_0; r \right] \delta \rho_2^0(r, t) dr \quad (2)$$

in which a is the mean radius of the Earth, r_{cmb} is the radius of the core–mantle boundary, and $\delta\rho_2^0$ is the time and radially varying harmonic coefficient of the (degree 2 and order 0) density perturbation within the Earth's mantle. G_2 is the kernel for the degree 2 nonhydrostatic geoid¹¹; it depends on the depth variation of relative viscosity $\nu(r)/\nu_0$, where ν_0 is an arbitrary reference scaling value. Our orbital calculations integrate backwards from the present day ($t = t_p$) configuration and we require changes to $\delta H(t)$ relative to the present value $\delta H(t_p)$. Accordingly, $\Delta H(t)$ will refer to $\delta H(t) - \delta H(t_p)$.

Our procedure for computing mantle-convection-induced perturbations $\Delta H(t)$ is as follows. (1) We use a model of the perturbations in seismic shear velocity in the mantle and convert this to an equivalent field of lateral temperature variations using an estimate of the (depth-dependent) temperature derivative of seismic shear wave velocity. (2) We next advect this temperature field backwards 20 Myr by neglecting thermal diffusion and viscous dissipation in the hydrodynamic equations governing our compressible-flow mantle model. The advection calculation is carried out by determining the kernels for the compressible-flow field¹¹ and then

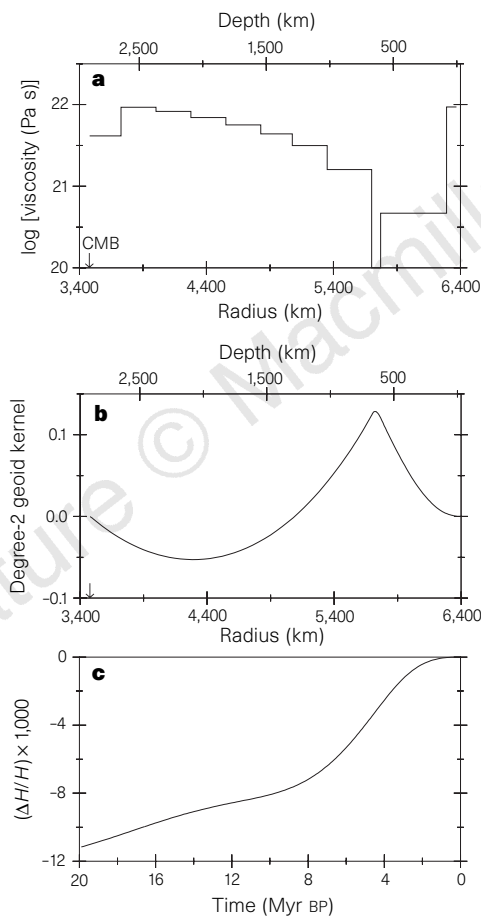


Figure 1 Predicting past variations in the Earth's dynamic ellipticity. **a**, The radial profile of mantle viscosity used in our reference (model A) viscous flow calculation^{19,22}. The low-viscosity zone above a depth of 670 km has a value of 4.5×10^{18} Pa s. **b**, Radially varying kernel for the flow-induced dynamic ellipticity (equation 2) computed using the relative viscosity variation associated with the profile in **a**. Our starting Earth model A is defined not only by the absolute viscosity in **a**, but also by the seismic heterogeneity model¹⁹ S.F1.K/WM13, and Karato's²⁰ velocity-to-density scaling profile. The density field is advected backwards in time using the procedure described in the text (the viscosity profile is assumed to remain constant with time). **c**, Predicted relative variation in the dynamic ellipticity over the past 20 Myr computed using equation (2) and the starting model A.

explicitly solving the conservation of energy equation¹². (3) Finally, we apply equation (2) to compute the time series $\Delta H(t)$. A similar philosophy of advecting density heterogeneities has been shown¹³ to predict a time series for convection-induced true polar wander which agrees with palaeomagnetic constraints. (An alternative approach to investigating long-term changes in the Earth's gravity potential, and hence the effect on the Earth's rotation, is offered by geological reconstructions of plate tectonic motions over the past 60–120 million years^{14–18}.)

Steinberger and O'Connell¹³ have justified, in detail, the validity of the backward-advection procedure adopted here. The accuracy of our calculations has also been checked by explicitly calculating the rate of change of temperature due to thermal conduction and viscous dissipation at $t = t_p$ and 20 Myr before present. In both cases this rate of change is less than 1% of the time variation in the laterally varying temperature field due to the advection process alone.

The advection of lateral temperature variations will be sensitive to the starting (present-day) Earth model. Accordingly, the starting model used in our reference calculation (Fig. 1a, b), henceforth model A, has previously been independently constrained to fit an extensive set of geophysical constraints. The seismic heterogeneity model, S.F1.K/WM13 (ref. 19), was derived via a joint inversion of seismic waveform data, differential travel times, normal-mode structure coefficients and a suite of geodynamic data. In combination with a laboratory-based estimate of the velocity-to-density scaling profile²⁰ and the viscosity variation shown in Fig. 1a, the model yields variance reductions better than 80% for both the present-day nonhydrostatic geoid and free-air gravity harmonics in the degree range 2–8 (ref. 19). Predictions based on the same

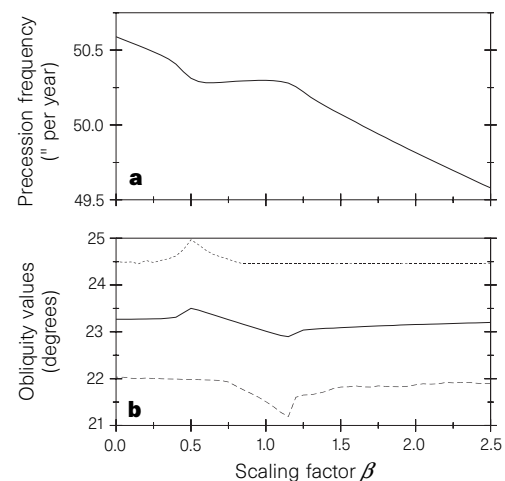


Figure 2 Precession and obliquity parameters determined from orbital integrations. Predicted **(a)** mean precession frequency and **(b)** maximum, minimum and mean obliquity derived from orbital integrations⁵ extending over the past 20 Myr. In the integrations, we adopt a sequence of perturbations to the dynamic ellipticity, $\beta \Delta H(t)/H$, where the time series $\Delta H(t)/H$ is shown in Fig. 1c and the constant β is varied from the value $\beta = 0$ (the nominal case of no convection-induced perturbation) to $\beta = 2.5$ in increments of 0.05. Following the suggestion by Laskar *et al.*⁵, the orbital integrations include a tidal dissipation term which is intermediate to the extremes of 0.0 and the present-day value (we therefore incorporate a dissipation rate equal to half the present-day value. This dissipation opposes the convection-induced passage into resonance. We neglect the effect of core–mantle friction which becomes significant only on very long timescales²⁵). In the absence of resonance effects, the perturbation to the nominal precession frequency, P_0 , would be equal to P_0 times the mean perturbation to the dynamic ellipticity. The latter perturbation is $-0.67 \times 10^{-2} \beta$ when the $\Delta H(t)/H$ profile in Fig. 1c is adopted, and hence the slope in Fig. 2a would, in this case, be about -0.34° per year. This value is in agreement with the background trend in the figure.

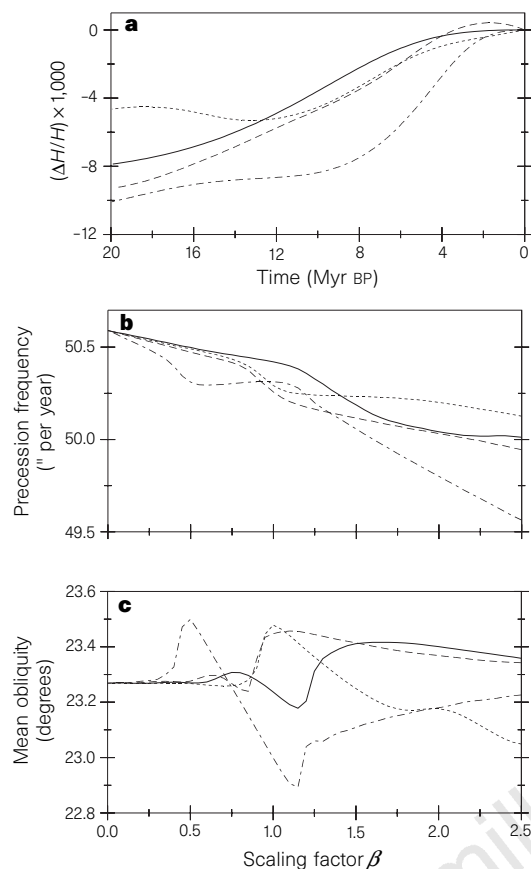


Figure 3 An Earth model sensitivity analysis. **a**, Predicted relative variation in the dynamic ellipticity over the past 20 Myr computed using equation (2) and four different starting Earth models. Model B (dashed-dotted line) is identical to model A (defined in Fig. 1) with the exception that the viscosity profile adopted in the calculation was derived from a nonlinear, iterative, joint inversion of satellite-derived long-wavelength free-air gravity anomalies up to degree and order 8 and an extensive set of post-glacial decay times from North America and Fennoscandia (see model MF2 of Mitrovica and Forte²²). In the case of model C (solid line) an absolute viscosity profile was adopted that satisfies surface observables associated with mantle convection alone²⁶; these include long-wavelength free-air gravity harmonics and plate motions. (Models B and C provide a variance reduction better than 80% for the present-day long-wavelength geoid harmonics when used in conjunction with Karato's²⁰ velocity-to-density scaling profile.) Earth models D (dashed line) and E (dotted line) differ from model A in the adopted three-dimensional seismic heterogeneity model. In particular, model D is based on a backward-advection of the Berkeley model SAW12D²⁷ whereas model E incorporates the Scripps model S16B30²⁸ as an initial condition. (Models D and E do not provide a good fit to the long-wavelength geoid when used with Karato's²⁰ velocity-to-density scaling profile. Accordingly, for these models we combine the adopted three-dimensional seismic heterogeneity model with a velocity-to-density scaling which yields a (least-squares) best fit to the geoid harmonics. In the case of model D, this scaling has a value of 0.10 in the upper mantle and 0.25 in the lower mantle. Analogous values for model E are 0.21 and 0.24.) As in Fig. 2, we have performed orbital integrations (which include tidal dissipation) using a sequence of time series $\beta \Delta H(t)/H$, for $0.0 < \beta < 2.5$, where $\Delta H(t)/H$ is given by each of the curves in **a**. The mean precession frequency and obliquity angle over the past 20 Myr derived from this exercise are shown in **b** and **c** respectively.

combination also fit the present-day degree-2 zonal components of both the satellite-observed nonhydrostatic geoid field and the geodetically inferred topography of the core–mantle boundary^{19,21}. The absolute viscosity scaling ν_0 (Fig. 1a) was obtained by seeking a best fit to a set of decay times determined from post-glacial sea-level curves collected from previously glaciated regions²².

The time series of $\Delta H(t)/H$ (Fig. 1c), computed by backward-advecting the starting model A, indicates that the dynamic ellipticity has increased by $\sim 1\%$ since the beginning of the Miocene as a consequence of mantle convection. The mean perturbation to the ellipticity, relative to the present day value, is about -0.67% .

We next performed many-body orbital integrations⁵ for the past 20 Myr. As input to these integrations, we adopted a suite of time series $\beta \Delta H(t)/H$ for the relative perturbation to the dynamic ellipticity, where $\Delta H(t)/H$ is given in Fig. 1c and β is a factor which is varied successively from 0 to 2.5. Summary results, shown in Fig. 2, show that the gradual convection-induced increase in the dynamic ellipticity indicated by our model (Fig. 1c) has led the Earth's precession and obliquity through a resonance over the past 20 Myr.

Inspection of Fig. 2a indicates that the resonance originates from an external forcing on the Earth with a frequency of about $50.3''$ per year. Laskar *et al.*⁵ have shown that the $s_6 - g_6 + g_5$ term, associated with the secular frequencies related to the perihelion of Jupiter and Saturn and the node of Saturn, has a frequency of $50.3021''$ per year (ref. 5), and this term is thus the source of the resonance. A departure from linearity in Fig. 2 occurs for β values in the range $0.3 < \beta < 1.5$. The broadness of this range, encompassing $\beta = 1$, suggests that our conclusion that mantle convection has led to a passage through resonance is remarkably robust.

In Fig. 3 we explore the issue of robustness in more detail by considering the sensitivity of our predictions to variations in the starting Earth model. Figure 3a shows time series of $\Delta H(t)/H$ for calculations in which either the radial viscosity profile or the model

of three-dimensional seismic heterogeneity (together with the associated, geoid-inferred, velocity-to-density scaling profile) is altered from the values adopted in model A. The mean perturbation over the past 20 Myr is -0.66% for model B and about -0.4% for models C, D and E. For each of the predictions in Fig. 3a we performed orbital integrations using the scaled time series $\beta \Delta H(t)/H$ ($0.0 < \beta < 2.5$), and summary results are given in Fig. 3b and c. (The use of this β scaling follows the approach of Laskar *et al.*⁵.) In all four cases the passage through the $s_6 - g_6 + g_5$ resonance is evident as the scaling factor β is increased. More importantly, the convection-induced ($\beta = 1$) changes in the dynamic ellipticity (the time series given in Fig. 3a) are, as in the case of model A (Figs 1 and 2), sufficient to perturb the Earth's precession and obliquity into the resonance regime.

Convection-induced variations in the dynamic ellipticity, whether they perturb the system into the $s_6 - g_6 + g_5$ resonance or not, have implications for past insolation variations on the Earth. Palaeoclimatic fluctuations are particularly sensitive to variations in insolation at high latitudes^{23,24} and in Fig. 4 we show four different predictions of summer insolation at 65° N for various time windows extending over the past 9 Myr. Lourens *et al.*²⁴ have compared insolation time series derived from orbital solutions with high-resolution measurements of sapropel (0–3 Myr BP) and carbonate (2.5–5.3 Myr BP) sedimentary cycles in Mediterranean marine sequences. On the basis of their comparison, reviewed in Fig. 4, they concluded that the nominal ($\beta = 0$) solution provided a better fit to the geological record than the maximal effect 'ice-age' model tested by Laskar *et al.*⁵.

The observational constraints described by Lourens *et al.*²⁴ are also satisfied by our orbital solutions which include the influence of convection-induced changes in the dynamical ellipticity (Fig. 4, models A and C). In fact, the insolation time series derived using models A–E are almost identical to the nominal case over the past 5 Myr. This agreement is due to the form of the predicted $\Delta H(t)/H$

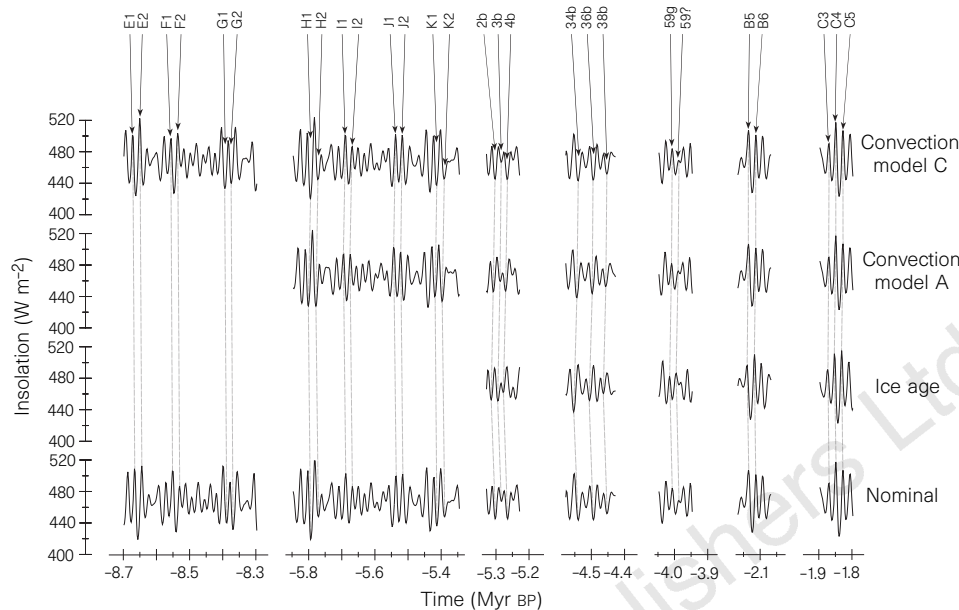


Figure 4 A comparison of predicted insolation time series. Mean summer (June–July) solar insolation at 65°N for various time ranges extending over the past ~9 Myr. The row labelled 'Nominal' represents the solution obtained by assuming no convection-induced perturbation to the dynamical ellipticity ($\beta = 0$). The time series labelled 'Ice age' was computed by reducing the dynamical ellipticity by a constant amount, equal to 0.23% of the observed present-day value, relative to the nominal orbital integration. This constant perturbation was investigated by Laskar *et al.*⁵ in order to consider the possible influence of ice-age induced perturbations to the dynamical ellipticity on precession, obliquity and insolation time series. Lourens *et al.*²⁴ compared the 'Nominal' and 'Ice age' time series to the observational record, and the labels 2b to C5 appearing between 5.35 and 1.8 Myr BP are adopted from their analysis. As an example, observed sapropel clusters C3–C5 and B5–B6 show, respectively, a thin-thick-thin and thick-thin alteration, and hence the insolation peaks correlated with the B5 and C4 clusters should be of

curves in Figs 1c and 3a. Model A (and also model B) yields a relatively small perturbation over the past 2–3 Myr and a rapidly increasing perturbation from 3 to 8 Myr BP. Indeed, the mean value of $\Delta H(t)/H$ over the past 8 Myr is a factor of 25 larger than the mean Quaternary (0–2 Myr BP) value for this model. In the case of model C (and also models D and E), the amplitude of the predicted $\Delta H(t)/H$ is small over the past 6 Myr and increases rapidly over the period from 6 to ~12 Myr BP. Thus, the influence of convection-induced changes in the dynamic ellipticity on the Earth's insolation time series should become pronounced near the Pliocene/Miocene boundary (~6 Myr BP), in the case of models A and B, and ~9 Myr BP for predictions based on models C, D and E. This point is confirmed in the time windows 5.9–5.4 and 8.7–8.3 Myr BP in Fig. 4.

As an example, the nominal prediction yields a distinct H1 layer in the carbonate record at 5.8 Myr BP (as does model C), whereas the model A solution predicts roughly equivalent H1 and H2 layers. Furthermore, the dominant I1 layer predicted by the nominal (and model C) solution contrasts with the more or less equivalent I1 and I2 layers arising from the model A prediction. In the case of the time window 8.7–8.3 Myr BP, the dominant F1 peak at ~8.55 Myr BP predicted in the nominal solution is not evident in the model C calculation.

Changes in the Earth's dynamic ellipticity associated with mantle convection are sufficient to have passed the orbital system through the $s_6 - g_6 + g_5$ resonance⁵ over the past 20 Myr. Predictions of high-latitude summer insolation that include convection dynamics begin to diverge from previously described solutions^{4,5,24} for times older than ~5.4 Myr BP. Convection-induced changes in the Earth's

larger magnitude than the adjacent peaks. In carbonate cycle 59 the appearance of a single grey layer in the sedimentary cores precludes more than one prominent insolation maximum in this cycle. Finally, distinct beige layers are found in the carbonate cycles 2b, 4b, 34b, 36b and 38b, and hence the associated minima in the insolation time series should be more prominent than in adjacent cycles. These requirements are consistent with the nominal orbital solution but they seem to rule out the 'Ice age' solution²⁴. The results labelled 'Model A' and 'Model C' represent solutions in which the time series of convection-induced perturbations $\Delta H(t)/H$ in Fig. 1c and Fig. 3a, respectively, are incorporated into the orbital integration. Insolation time series for models D and E (not shown) are similar to those for model C, whereas the insolation time series derived using model B (also not shown) closely matches the 'Model A' result. The labels E1–K2 are introduced here solely for the purposes of the discussion in the text. All orbital integrations incorporate the tidal dissipation rates described in Fig. 2.

obliquity and precession will have a measurable effect on sedimentary cycles and climate proxy records (such as oxygen isotope and sea-surface temperature records) once the time range of these observational constraints is increased to encompass the Pliocene/Miocene boundary. □

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Regeneration of adult axons in white matter tracts of the central nervous system

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It is widely accepted that the adult mammalian central nervous system (CNS) is unable to regenerate axons¹. In addition to physical or molecular barriers presented by glial scarring at the lesion site^{2–4}, it has been suggested that the normal myelinated CNS environment contains potent growth inhibitors^{5,6} or lacks growth-promoting molecules^{1,7}. Here we investigate whether adult CNS white matter can support long-distance regeneration of adult axons in the absence of glial scarring, by using a microtransplantation technique⁸ that minimizes scarring⁹ to inject minute volumes of dissociated adult rat dorsal root ganglia directly into adult rat CNS pathways. This atraumatic injection procedure allowed considerable numbers of regenerating adult axons immediate access to the host glial terrain, where we found that they rapidly extended for long distances in white matter, eventually invading grey matter. Abortive regeneration correlated precisely with increased levels of proteoglycans within the extracellular matrix at the transplant interface, whereas successfully regenerating transplants were associated with minimal upregulation of these molecules. Our results demonstrate, to our knowledge for the first time, that reactive glial extracellular matrix at the lesion site is directly associated with failure of axon regrowth *in vivo*, and that adult myelinated white matter tracts beyond the glial scar can be highly permissive for regeneration.

Previous studies have demonstrated that embryonic axons can grow long distances within adult CNS white matter^{8–10}, but it was considered that embryonic neurons might possess a superior intrinsic axon growth potential compared with adult neurons, or

that they had not yet acquired receptors for myelin inhibitors (M. E. Schwab, personal communication). Thus, the question remained as to whether adult white matter could be an inherently conducive highway for axon regeneration from an adult neuron in the absence of glial scarring. We used a microtransplantation technique⁸ to address this question by injecting 0.5- μ l volumes of dissociated suspensions of either adult or postnatal-day-eight (P8) dorsal root ganglia (DRG) neurons and accompanying satellite cells directly into the white matter of the corpus callosum ($n = 25$) or the fimbria ($n = 16$) (Fig. 1). Transplanted DRG neurons were used for the following reasons: (1) a subpopulation maintains high levels of calcitonin-gene-related peptide (CGRP) following transplantation and can be readily distinguished from the CGRP-negative axons of the host corpus callosum and fimbria; (2) DRG neurons (including the CGRP-containing population) will not regenerate their axons within the CNS beyond the second postnatal day^{11,12}; (3) examination of transplanted neurons avoids the complications of axonal sparing associated with crush or partial injury paradigms; and (4) it has been shown that DRGs are inhibited *in vitro* by mature oligodendrocytes or CNS myelin components^{13,14}, and our observations verify this finding for the adult CGRP-positive subpopulation (data not shown).

Fluorescent immunolabelling for CGRP at 2 days ($n = 4$ adults) post-transplantation showed intense staining of a subset of DRG neuronal cell bodies which had extended two or three minor processes ($<20 \mu\text{m}$) that resembled filopodia and one longer axonal process with a growth cone of simple morphology. Axons

Table 1 No. of adult donor CGRP⁺ neurons and their regenerating axons

Survival time	Identification no.	A	B	C
2 days	V49	60	2	0
	V50	51	0	0
4 days	V9	108	39	15
	V10	130	41	16
	V11	75	28	10
	V51	117	35	12
6 days	V43	110	38	32
	V44	116	40	31
	V54	94	32	27
	V55	81	27	21

Contralaterally directed regenerating axons from individual successful intracallosal grafts were counted through adjacent 60- μm horizontal sections within callosal white matter at the midline, and from the contralateral rostrum callosal white matter at its interface with overlying cortical grey matter. Columns: A, numbers of CGRP⁺ neuronal cell bodies in intracallosal grafts; B, numbers of axons at midline of corpus callosum; C, numbers of axons at contralateral white/grey matter interface. Average number of CGRP⁺ neuronal cell bodies, 94; average number of axons at midline, 35 (4- and 6-day combined); average number of axons at contralateral white/grey matter interface at 2 d, 0; at 4 d, 13.3; at 6 d, 27.7.

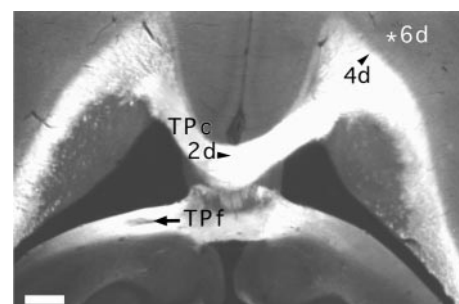


Figure 1 Photomicrograph of an unstained 60- μm horizontal section through an adult rat brain indicating microtransplant locations and maximum distances for adult axon regeneration from intracallosal grafts at 2 (2d, arrowhead), 4 (4d, arrowhead) and 6 (*6d) days post-transplantation. The ovoid transplants were $\sim 1 \text{ mm}$ long by 0.5 mm wide. The light-reflective properties of the myelinated white matter tracts contrast with the transplant seen easily in the fimbria in this section. TPc, transplant position in corpus callosum. TPf (arrow), transplant position in fimbria. Scale bar, 1 mm .

varied in length, some having already entered the host tract as far as 0.5 to 2 mm away from the edge of the transplants, whereas others had only ventured little more than 100 μm into the host white matter. By 4 days post-transplantation ($n = 18$ transplants total), large numbers of CGRP-positive axons from P8 ($n = 6$ successful transplants) and adult ($n = 7$ successful transplants) DRG microtransplants crossed the graft/host interface and extended contralaterally for maximum distances of ~ 6 mm (Figs 1, 2a). Axons always exited in about equal numbers from clearly defined depar-

ture points at either pole of the ovoid-shaped transplants which were in continuity with host-tract glial structures (Fig. 3a, c). Once out of the graft territory and into host white matter, donor axons did not appear to fasciculate with each other and lacked collaterals (Fig. 2a). Regenerating axons exhibited 'bullet-shaped' growth cones (Fig. 2b) with a single leading filopodium, resembling those previously observed within developing CNS pathways where axons were growing in a rapid and uninhibited fashion¹⁵. Double staining for CGRP and a myelin-specific antigen showed that the growing

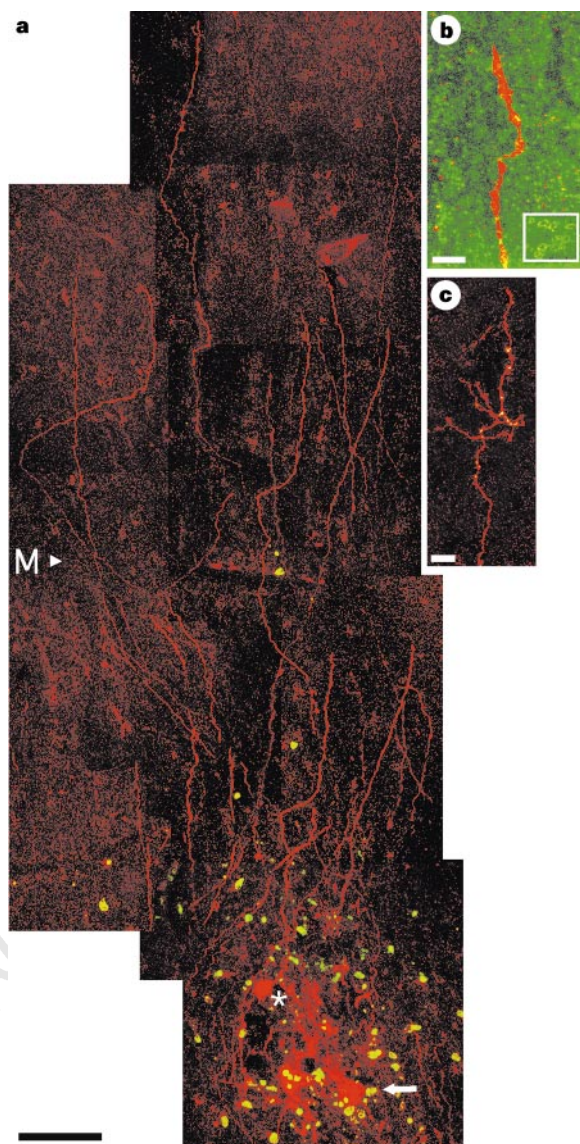


Figure 2 Regenerating adult DRG axons in adult rat corpus callosum. **a**, Confocal montage of a portion of an intracallosal adult DRG microtransplant scanned through two adjacent 60- μm horizontal sections. CGRP⁺ donor adult axons (red) exit the graft and extend within host white matter to cross the midline (M, arrowhead) to enter the contralateral hemisphere. Satellite cells (p75; green) were associated with donor neuron cell bodies (an example is indicated by the arrow) but not with axons. The asterisk indicates a CGRP⁺ donor neuron near the transplant interface with host white matter (see also Fig. 3c). Survival, 4 days; scale bar 100 μm . **b**, High-power confocal image through 17 μm of tangentially sectioned tissue showing a CGRP⁺ (red) adult donor axon with a streamlined growth cone extending through the myelin-rich (CNS myelin-specific mAb 328; green) adult host callosum, 2.5 mm from the graft. Inset: low-power image of mAb 328 immunohistochemistry shows typical myelin rings when white matter is cut in cross-section. Survival, 4 days; scale bar, 10 μm . **c**, Confocal image of a donor adult CGRP⁺ axon terminal within host cortical grey matter. Survival, 6 days; scale bar, 50 μm .

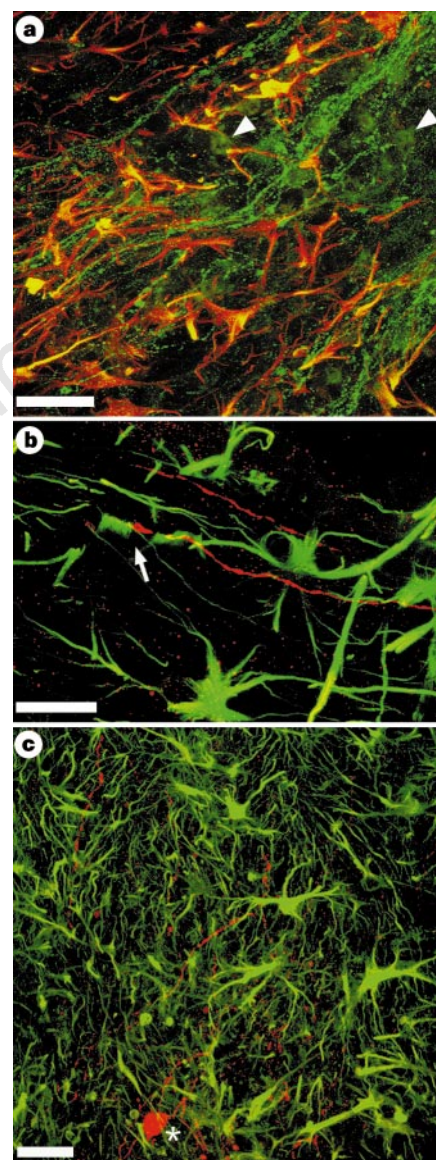


Figure 3 Transplant integration and association of regenerating axons with host astrocytes. **a**, Confocal image (60 μm) showing regenerating CGRP-positive P8 axons (green) associated with GFAP-positive astrocytes (red) in the adult fimbria. Large numbers of axons are exiting the transplant (top right) and growing in close association with astrocyte processes in white matter. Arrowheads indicate examples of faintly stained CGRP-positive neuron cell bodies. Survival, 4 days; scale bar, 20 μm . **b**, Confocal image (20 μm) showing adult donor DRG CGRP-positive axons (red) extending through white matter of the adult host fimbria. A growth cone of a regenerating axon (arrow) is closely aligned with the GFAP-positive (green) longitudinal processes of the host adult white-matter astrocytes. Survival, 2 days; scale bar, 20 μm . **c**, Confocal image (60 μm) of the same adult donor intracallosal transplant shown in Fig. 2a restained for GFAP (green). Asterisk marks the same CGRP-positive neuron (red) shown in Fig. 2a. The transplant is completely integrated with no readily definable interface between the graft and host tissue. Survival, 4 days; scale bar, 50 μm .

tips of the adult axons were extending through white matter with a normal myelin content (Fig. 2b). At 6 days ($n = 6$ successful transplants), donor axons could be seen exiting the callosum in both hemispheres to enter host prefrontal cortex, a distance contralaterally of 6–7 mm from the transplant boundary (Fig. 1) and 2–3 mm ipsilaterally. Some of the donor axons had now branched to form simple terminal arbors, suggesting innervation of host grey matter (Fig. 2c). Putative regenerated terminals were confirmed to be continuous with axons leading back to the transplant. Counts of CGRP-positive neurons and their axons within host white matter from successful adult donor grafts at 4 and 6 days combined show that on average 35% of CGRP-positive neurons had projected axons across the midline of the callosum (Table 1). Furthermore, 80% of these axons at the midline had reached the contralateral white–grey matter interface of the rostrum of the corpus callosum at 6 days (Table 1). Ipsilaterally projecting axons were not quantified owing to the close proximity of some grafts to ipsilateral host grey matter. Control sections from normal animals ($n = 2$), or those with only micropipette stabs ($n = 2$), showed no immunoreactivity for CGRP in the callosum or fimbria. No differences were observed in either the speed or extent of regenerative growth achieved by adult or P8 donor axons, results that are virtually identical to those described for embryonic CNS neurons microtransplanted in a similar fashion^{8,9}.

At 8 days survival ($n = 11$), CGRP immunoreactivity of axons and cell bodies had downregulated in all transplants. Immunostaining for peripherin showed that significant numbers of DRG neurons were still present within the transplant neuropil, suggesting that CGRP downregulation may be an active rather than a degenerative process. Peripherin immunohistochemistry could not be used as an unequivocal marker of regenerating DRG axons because of overlap with staining of resident axons in the forebrain.

Immunohistochemistry (using an antibody raised against antigen p75) for satellite cells¹⁶ accompanying the donor DRG neurons showed no apparent association between these cells and CGRP-positive donor axons, as the growing tips of axons were far in

advance of these slowly migrating glia in host white matter (Fig. 2a). Therefore it is unlikely that the satellite cells are modifying the entire length of the CNS pathways to make them more permissive for axon regeneration. However, the satellite cells may be critical for inducing regenerative outgrowth from transplants by modifying the graft/host interface, or by augmenting trophic support of the regenerating neurons. Reducing satellite cells to lower levels still allowed for robust DRG regeneration, and even at high levels their presence was insufficient to promote axon growth in those transplants that failed to regenerate. DRG neurons were only weakly immunoreactive for p75 (ref. 17).

Transplants exhibiting robust axon regeneration into adult white matter at 2, 4 and 6 days post-transplantation had very low levels of chondroitin-6-sulphate proteoglycan in matrix at the graft/host interface, with only light staining surrounding donor neuronal cell bodies (data not shown). There was a striking upregulation of proteoglycan staining at the boundaries of those transplants ($n = 7$) whose axons failed to enter the adjacent host white matter. Double staining for CGRP and proteoglycans in these transplants at 4 and 6 days showed axons that had stopped within the proteoglycan-rich boundaries or had actively turned away from the boundary and looped back into the transplant interior (Fig. 4a). A number of proteoglycans inhibit axon growth *in vitro*^{18,19} and *in vivo* their upregulation coincides developmentally with the age beyond which the CNS fails to support axon regeneration^{20,21}. These previous observations, taken together with our results, strongly suggest a potent role for reactive extracellular matrix in causing regeneration failure in the adult brain. However, we do not know at present which specific components of the matrix are crucial for axon repulsion. There are many different types of inhibitory proteoglycans²² and in addition there may be other classes of inhibitors^{23,24} co-localized within the barrier.

The molecules that trigger upregulation of proteoglycan-rich matrix are unknown and their characterization may help in developing therapeutic interventions. The smooth-edged, oval geometry of the proteoglycan border around failed transplants, coupled with

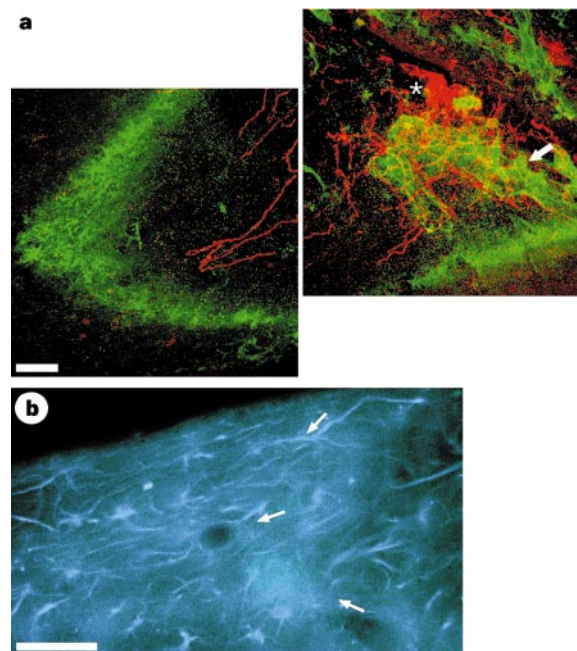


Figure 4 Regeneration failure associated with a proteoglycan boundary in the absence of a physical glial scar. **a**, Confocal images from the same 60- μ m section of an adult intrafimbrial transplant that failed to regenerate. CGRP-positive axons (red) grew within the transplant but did not cross the proteoglycan-rich extracellular matrix boundary (green) which encapsulates the transplant. Proteoglycan was also deposited around blood vessels but only within the

transplant (arrow). Asterisk denotes CGRP⁺ neuron cell bodies. Survival, 4 days; scale bar, 50 μ m. **b**, Fluorescent photomicrograph (not confocal) of the 60- μ m section shown in **a**, immunostained for GFAP (AMCA; blue). Astrocytes are aligned and continuous across the graft/host interface; their morphology does not predict the location of the inhibitory proteoglycan boundary (arrows). Scale bar, 100 μ m.

the presence of reactive, matrix-coated vessels within these grafts (Fig. 4a), suggests that a local breakdown of the blood-brain barrier precedes regeneration failure. The resulting intraparenchymal diffusion of serum borne factors could, in turn, trigger the production of inflammatory cytokines²⁵, as well as the reactive glial response leading to increased matrix production²⁶.

Staining for glial fibrillary acidic protein (GFAP) showed that in all transplants, even those with little or no outgrowth of axons across the proteoglycan-rich boundary, longitudinal astrocytic processes were aligned and in continuity from the host tract deep into the transplant neuropil (Figs 3c, 4b). We have also found that proteoglycans are distributed precisely in the region of neurite dystrophy observed in the microlesion model of axon-regeneration failure (unpublished observations), where cut adult axons again fail to regenerate in the presence of a continuous aligned astroglial pathway²⁷. Thus, in two different *in vivo* models, failure of regeneration was associated with axon-growth arrest at proteoglycan-rich boundaries, rather than at a physical barrier presented by host astrocytes.

Although oligodendrocytes produce factors capable of inhibiting their intrinsic potential to promote axon growth on their own surfaces, the question has remained as to whether they can mask the growth-promoting potential of all other cell types *in vitro*^{14,28,29} or *in vivo*. The overall geometry of the intratract astroglia closely paralleled the trajectories of the regenerating axons, suggesting that the intrafascicular astroglia can overcome the inhibitory influence of myelin, at least under the present circumstances (Fig. 3b).

So far we have characterized only one type of neuron in our adult-to-adult transplant model. Intrinsic limitations may contribute to the poor regenerative response of certain subpopulations of mature CNS neurons when confronted with a growth-promoting environment³⁰. Irrespective of whether other types of adult neuron can regrow axons in a similar fashion once microtransplanted, we have demonstrated that, once beyond the molecular repulsion associated with a forming glial scar, the adult CNS environment can support regenerative growth from a type of adult neuron that has never before been shown to possess such a remarkable ability to regenerate its axon within adult CNS white matter. □

Methods

Dissociation of dorsal root ganglion neurons. Separate single-cell suspensions of DRG were prepared from adult (250–300 g) and P8 Sprague-Dawley rats. Lumbar and cervical DRGs were dissected, capsules removed, incubated in dispase (5 mg ml⁻¹) and collagenase (100 mg ml⁻¹), and resuspended in 50–100 µl L15 both with CO₂ for maximum cellular density. In some experiments, dissociated cells were preplated to remove adherent non-neuronal cells, and the neuron-enriched supernatant was plated on polylysine/laminin-coated culture plastic overnight in L15-CO₂ with 5% rat serum. Adherent neurons were resuspended in 50–100 µl L15-CO₂, yielding a concentrated and purified neuronal suspension ~1,000 neurons per µl.

Surgical microtransplantation. Adult Sprague-Dawley female rats (200–225 g) were anaesthetized with intramuscular ketamine (100 mg kg⁻¹) and xylazine (2.4 mg kg⁻¹). Stereotactic surgery was done as described⁹. Cell suspensions (0.5 µl) were slowly injected with a picospritzer (General Valve) through a glass micropipette with a bevelled tip and outer and inner diameters of 90 and 70 µm respectively. Thirty-four microtransplants were injected using adult DRG cell suspensions, twenty-six of which contained neurons with normal numbers of satellite cells and eight having small numbers of satellite cells. Seven microtransplants of DRG suspensions from P8 rats with normal satellite cell numbers were also injected. Microtransplants that missed white matter tracts (*n* = 4) were excluded from analysis. Unoperated animals (*n* = 2) and animals with micropipette stabs only (*n* = 2) were used as controls for endogenous CGRP staining in host brains. Following post-operative periods of 2, 4, 6 and 8 days, animals were transcardially perfused with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde in PBS. Dissected brains were postfixed in 4% paraformaldehyde, and 60-µm Vibratome sections were cut in a modified horizontal plane (parallel to the surface of the cortex) before being

processed for immunohistochemical analysis.

Immunohistochemistry. Sections were washed in PBS, blocked with 4% goat serum with 0.1% Triton X-100 in PBS, and incubated overnight with primary antibody in blocking solution followed by secondary and tertiary steps for single, double and triple staining by standard immunocytochemical methods. A polyclonal antibody against CGRP (Accurate; 1:350) was used to identify a subset of DRG neurons. Antibodies against the following antigens were also used: GFAP (Sigma; 1:100), p75 (Boehringer Mannheim; 1:100), myelin/oligodendrocytes (mAb 328; Chemicon; 1:100), chondroitin-6- and -4-sulphate proteoglycans (CS56; Sigma; 1:100), peripherin (Boehringer Mannheim; 1:350). Fluorochromes used to visualize antibody labelling included FITC, Oregon Green, Cy3 and AMCA. Stained sections were examined using a Zeiss laser scanning confocal microscope and a Leitz Orthoplan-2 fluorescence light microscope.

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The cellular prion protein binds copper *in vivo*

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The normal cellular form of prion protein (PrP^C) is a precursor to the pathogenic protease-resistant forms (PrP^{Sc}) believed to cause scrapie, bovine spongiform encephalopathy (BSE) and Creutzfeldt–Jakob disease¹. Its amino terminus contains the octapeptide PHGGGWGQ, which is repeated four times and is among the best-preserved regions of mammalian PrP^C. Here we show that the amino-terminal domain of PrP^C exhibits five to six sites that bind copper (Cu(II)) presented as a glycine chelate. At neutral pH, binding occurs with positive cooperativity, with binding affinity compatible with estimates for extracellular, labile copper. Two lines of independently derived PrP^C gene-ablated (*Prnp*^{0/0}) mice exhibit severe reductions in the copper content of membrane-enriched brain extracts and similar reductions in synaptosomal and endosome-enriched subcellular fractions. *Prnp*^{0/0} mice also have altered cellular phenotypes, including a reduction in the activity of copper/zinc superoxide dismutase and altered electrophysiological responses in the presence of excess copper. These findings indicate that PrP^C can exist in a Cu-metalloprotein form *in vivo*.

Our copper binding experiments differ from those of earlier studies^{2,3} with regard to three considerations. First, the protein fragment used in our studies consists of the entire N-terminal domain of human PrP expressed in *Escherichia coli* (residues 23–98). Second, because free copper has a tendency to hydrolyse in neutral solution to form metal-hydroxy and metal-oxy polymers, which may be kinetically inert or may bind to proteins in a nonspecific manner, Cu(II) was supplied as a Cu-glycine₂ complex, which is stable at neutral pH. Additional advantages of this approach are that it mimics the *in vivo* situation and, because glycine competes for free copper, spurious binding to low-affinity sites is suppressed. Third, potential interactions between copper and the buffer system were reduced by using *N*-ethylmorpholine-HCl (pH 7.4), a tertiary amine thought to possess minimal copper-chelating activity⁴.

Once we had confirmed that PrP23–98 remained soluble in the presence of excess of Cu(II), mixtures of PrP23–98, copper-glycine₂, buffer and KCl were placed in a dialysis chamber and allowed to equilibrate with the contents of a second chamber containing only buffer and salt. Copper content at equilibrium in the protein-containing and blank chambers was determined by atomic absorption spectroscopy. In a saturation plot of [bound Cu]/[total PrP23–98] versus [free Cu] (Fig. 1a), saturation was reached at 5.6 mol equivalents of Cu (range 5.2–6.0, at 18-fold molar excess). Binding data were subjected to graphical ‘Scatchard’ analysis⁵. The resulting convex plot indicated positive cooperativity (Fig. 1b). Scatchard analysis is designed to yield numbers and stability of multiple equivalent binding sites and a curvilinear plot indicates unequal binding-site affinities. To gain insight into the cooperative nature of

the multiple PrP23–98 binding sites, a Hill plot was constructed. The slope at the midpoint of the plot defines a Hill coefficient, that is the number of Cu(II) ions added on average in a cooperative fashion, of 3.4 (Fig. 1c). Half-maximal binding was obtained at 5.9 μ M glycine-chelated copper. Parallel binding studies of bovine serum albumin (BSA) versus copper-glycine yielded a Scatchard plot with intercepts indicative of between 1 and 1.6 high-affinity binding sites per molecule, in accord with the literature for copper binding to BSA^{4,6,7}. It is therefore unlikely that the results obtained with PrP23–98 reflect errors in the binding studies.

To test the hypothesis that PrP^C binds copper *in vivo*, total-reflection X-ray fluorescence was used to quantify the wet weight content of copper, iron and zinc ions in blood serum, and membrane/organelle-rich fractions of brain, liver, kidney and muscle from wild-type and *Prnp*^{0/0} mice (Table 1). Wild-type mouse brains have a much higher level of membrane-associated copper than *Prnp*^{0/0} mice, but no significant difference could be seen for zinc. A significant difference was noted in the levels of copper and zinc in the liver and in the concentration of iron in the kidney. Serum from *Prnp*^{0/0} mice showed a significant increase in copper content. Measurements of caeruloplasmin levels⁸ showed that serum from wild-type mice contained 25.0 ± 4.8 units ml⁻¹ ($n = 4$), not significantly different from the content of *Prnp*^{0/0} mice (21.3 ± 3.2 units ml⁻¹; $n = 4$). Furthermore, in ‘ashed’ tissue samples and using atomic absorption spectroscopy the same tendency for

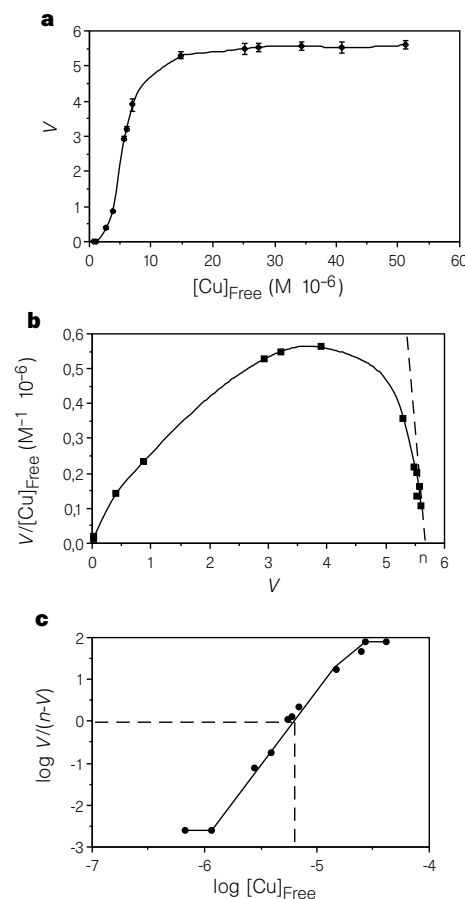


Figure 1 Cu-binding properties of PrP23–98. All experiments represent equilibrium dialysis analyses performed in 25 mM *N*-ethylmorpholine (pH 7.4), 150 mM KCl, at 4°C. Copper was presented as a Gly:Cu:Gly chelate. **a**, Saturation plot of V (moles Cu bound per mole of protein) versus total ‘free’ Cu measured at 2.7 μ M PrP23–98. Error-bars represent standard deviations. **b**, Scatchard plot; n is the number of binding sites. **c**, Hill plot calculated assuming $n = 5.6$. The slope of the mid-portion of the graph is 3.4.

low copper content in brain samples from *Prnp*^{0/0} mice was noted, with values of 193 ± 16 ($n = 4$) and 16.2 ± 2 ($n = 4$) μg per g dry weight in wild-type and *Prnp*^{0/0} mice, respectively. This tendency was recapitulated in synaptosomes and endosomal enriched fractions isolated after step-gradient centrifugation in the presence of Percoll and sucrose. Both synaptosomes and endosomes from wild-type mice showed a tenfold higher copper content than *Prnp*^{0/0}

Table 1 Divalent cations in membrane-rich extracts from mouse

Organ	Cu	Fe	Zn
Brain			
Wild type ($n = 8$)	20.1 ± 1.4	5.2 ± 0.3	5.0 ± 0.1
<i>Prnp</i> ^{0/0} ($n = 12$)	$1.3 \pm 0.05^*$	5.7 ± 0.9	5.6 ± 0.3
Liver			
Wild type ($n = 8$)	2.23 ± 0.2	37.4 ± 4	11.7 ± 0.9
<i>Prnp</i> ^{0/0} ($n = 12$)	$1.47 \pm 0.2^*$	29.8 ± 6	$8.6 \pm 0.75^*$
Kidney			
Wild type	1.5 ± 0.1	15.7 ± 2.0	7.2 ± 0.5
<i>Prnp</i> ^{0/0}	1.6 ± 0.1	$22.2 \pm 2.9^*$	6.6 ± 0.4
Muscle			
Wild type	0.5 ± 0.1	5.1 ± 1.2	3.5 ± 1.1
<i>Prnp</i> ^{0/0}	0.5 ± 0.1	4.4 ± 1.3	4.7 ± 1.3
Serum			
Wild type	0.56 ± 0.05	8.8 ± 3.0	1.24 ± 0.11
<i>Prnp</i> ^{0/0}	$0.91 \pm 0.13^*$	5.1 ± 0.8	0.91 ± 0.09

Values are expressed as μg cation per g wet weight of membrane, except serum which is expressed as μg cation per ml serum. Determinations were made using the TXRF method²⁷. All values are mean $\pm$ s.e. for at least 4 mice.

*Values for which there is a significant (Student's *t*-test, $P < 0.05$) difference between the *Prnp*^{0/0} value and the corresponding wild-type value.

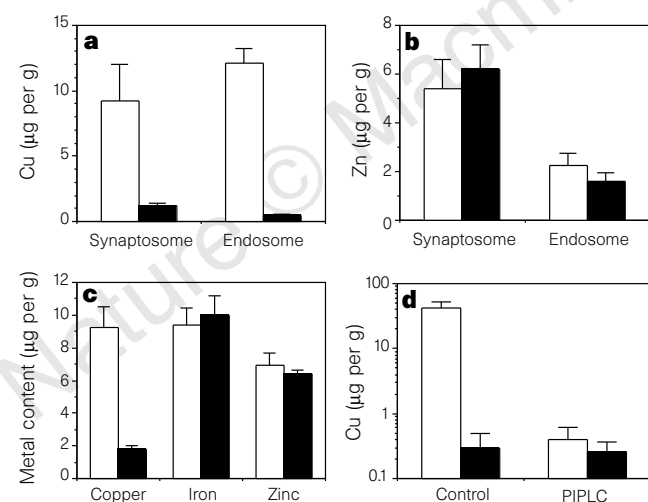


Figure 2 Assessment of copper content. TXRF was used to quantify the divalent cations in subcellular fractions of brain. Values shown are for wet weight samples. Open bars, wild type; black bars, *Prnp*^{0/0}. **a**, The copper content of subcellular fractions was found to be significantly higher in both synaptosomal and endosomal fractions of wild-type mice (Student's *t*-test, $P < 0.05$). **b**, The zinc content of subcellular fractions of wild-type and *Prnp*^{0/0} mice showed no significant difference. (Student's *t*-test, $P > 0.05$). **c**, The copper, zinc and iron content of membranes from the brains of a second strain of adult *Prnp*^{0/0} and wild-type mice with the same genetic background (129/Ola) (ref. 9) determined using TXRF analysis. *Prnp*^{0/0} mice had lower levels of copper, but not zinc or iron, than wild-type mice. **d**, TXRF analysis of membrane fractions from cultured cerebellar cells shows a much higher concentration of copper in wild-type cell membranes than in *Prnp*^{0/0} cells. Treatment for 3 h of these cell cultures with 0.2 U ml^{-1} phosphatidylinositol-specific phospholipase C (PI-PLC), a lipase known to cleave PrP^{C} from the cell membrane, results in a reduction in the membrane copper content of wild-type cells (Student's *t*-test, $P < 0.05$), the copper content of *Prnp*^{0/0} cells remains unchanged. Mean and s.e. are shown for 3–4 experiments.

fractions (Fig. 2a). There was no significant difference in zinc content between wild-type and *Prnp*^{0/0} mice (Fig. 2b).

Total reflection X-ray fluorescence (TXRF) analysis of membrane copper, zinc and iron content in an independently derived *Prnp*^{0/0} mouse strain⁹ and the appropriate wild-type mice (129/Ola) revealed a similar deficit in copper (Fig. 2c). Taken together, these results provide strong evidence that cell membranes deficient in PrP^{C} are also deficient in copper.

Phosphatidylinositol-specific phospholipase C (PI-PLC) cleaves PrP^{C} from the cell surface¹⁰, so we used TXRF to investigate the effects of PI-PLC on the copper content of membranes from primary cultures of cerebellar cells. Before PI-PLC treatment, we observed a great reduction in the copper content of *Prnp*^{0/0} cells (Fig. 2d). PIPLC treatment specifically reduced the copper content of wild-type fractions, but had no effect on the copper content of *Prnp*^{0/0} cell fractions.

As the synaptosomal copper content in wild-type mice is high (Fig. 2a), copper and PrP^{C} may be required for synaptic transmission, as suggested previously¹¹. Slice preparations of the cerebellum from wild-type and *Prnp*^{0/0} mice of 9–12 days old were analysed using the patch-clamp technique to monitor inhibitory postsynaptic currents (IPSCs) in Purkinje cells. No difference was observed in the normalized sum of amplitudes after application of $2 \mu\text{M}$ CuSO_4 on Purkinje cells of wild-type mice ($n = 5$) (Fig. 3a), but in *Prnp*^{0/0} mice this copper concentration led to a significant reduction of

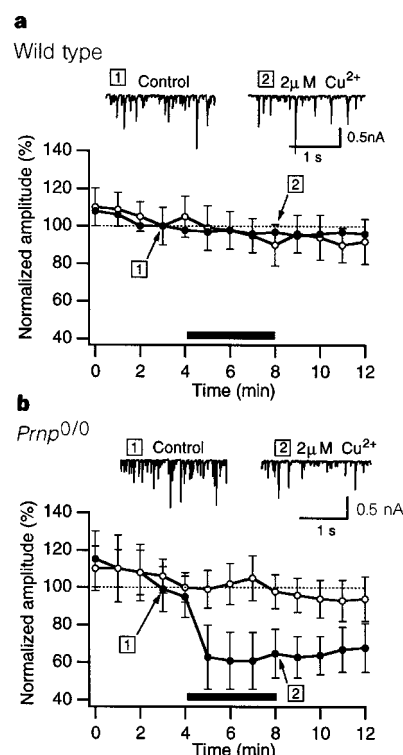


Figure 3 Effect of copper on spontaneous inhibitory synaptic currents in cerebellar Purkinje cells in *Prnp*^{0/0} and wild-type mice. **a**, Application of $2 \mu\text{M}$ CuSO_4 (filled circles) in wild-type Purkinje cells does not change the sum of IPSC amplitudes in comparison to untreated control (open circles). **b**, in *Prnp*^{0/0} Purkinje cells the sum of IPSC is significantly reduced by $2 \mu\text{M}$ CuSO_4 (filled circles). Each point represents the mean $\pm$ s.e.m. of sum of amplitudes of all synaptic events $>20 \text{ pA}$ during 60-s intervals normalized to the values before application of copper. Representative current traces from one experiment in wild-type (**a**) and *Prnp*^{0/0} mice (**b**) are taken before (control) and 4 min after application of $2 \mu\text{M}$ Cu^{2+} . The traces in the inserts were recorded at the time points indicated by the arrows. Bar, time of CuSO_4 application. Mean and s.e. are shown for 5 experiments.

IPSC amplitudes in Purkinje cells ($n = 5$) (Fig. 3b). Thus the loss of copper-binding capacity in *Prnp*^{0/0} membranes may expose synaptic transmission to alterations in the presence of copper that would be sequestered or taken up by PrP^C in wild-type cells¹².

A robust measure of binding ability is given by the copper concentration needed for 50% saturation. Because free Cu(II) rarely exists, it is more reasonable to consider chelated forms that exist in physiological settings. Total Cu(II) is estimated at 16–20 μM in the blood, between 0.5–2.5 μM in the cerebrospinal fluid, and 15 μM in the synaptic cleft^{13,14}. More than 60% is bound to caeruloplasmin and the remainder is complexed with albumin, transcuprein, peptides and amino acids¹³, which are thought to constitute the accessible pool of copper for most tissues^{15,16}, so candidate cell-surface binding proteins such as PrP^C must be able to compete Cu(II) from such ligands. This is the case for PrP23–98 and Cu(II)-glycine₂, where half-maximal binding occurs at 5.9 μM . For Cu(II)-histidine₂ (which forms a stronger Cu(II) complex than glycine), half-maximal cooperative binding occurs at 2.5 μM with a stoichiometry of 3.0 mol Cu(II) per mol protein at saturation (data not shown). The dissociation constants of Cu(II)-glycine₂ and Cu(II)-histidine₂ to ionic metal and two molecules of amino acid are $\sim 10^{-15}$ and 10^{-18} , respectively¹⁷. Thus the concentration of ionic Cu(II) present in micromolar solutions of metal/amino-acid chelate (and hence half-maximal binding to PrP23–98 expressed in terms of ionic metal) can be calculated to be subnanomolar. The avidity of BSA for Cu(II)-glycine, measured in parallel to PrP23–98, is greater but in the same range (K_d for high-affinity site $\sim 0.25 \mu\text{M}$), and it is notable that dissociation constants of 10^{-11} and 10^{-16} M have been reported for BSA/ionic copper¹⁷.

We conclude that PrP^C is either a principal copper-binding protein in brain membrane fractions, or that it controls the activity of other membrane-associated copper-binding proteins. Although we cannot exclude the latter possibility, the release of copper from the membranes of cerebellar cells by PI-PLC would demand that hypothetical copper-binding proteins regulated by PrP^C either possess GPI anchors themselves or are tethered to membranes through an interaction with PrP^C. Instead, half-maximal binding by PrP23–98 to glycine- and histidine-chelated copper at micromolar concentrations similar to those determined for chelated copper in biological fluids argues that PrP^C is a copper-binding metalloprotein. Extrapolating from the cooperative binding of O₂ to haemoglobin, medium-affinity cooperative binding of Cu(II) to PrP^C may allow rapid exchange of Cu(II) with other transport molecules. Thus PrP^C may serve as a 'sink' to trap ionic Cu(II) and/or as a shuttle to regulate the Cu(II) content of intracellular compartments, perhaps explaining the reduced activity of the copper-dependent enzyme superoxide dismutase-1 (SOD-1) in *Prnp*^{0/0} mouse brains¹⁸. It is not known whether Cu(II) bestows structural or catalytic properties upon PrP^C, but conformational alterations on addition of Cu(II) have been reported^{3,19,20}, perhaps cooperative binding maximizes occupancy and so amplifies the conformational shifts accompanying Cu-binding. Binding of transition metals by PrP^C may also offer insights into the pathogenic mechanisms of prion disease²¹. Our results indicate that prion protein is a cuproprotein, like monoamine oxidase, APP and SOD-1, which are implicated in the pathogenesis of Parkinson's disease, Alzheimer's disease, and familial amyotrophic lateral sclerosis. Although mechanisms differ for these diseases, the common denominator of cuproproteins indicates that neurons are sensitive to perturbations in copper physiology and suggests that they may possess special mechanisms—presumably including PrP^C—to regulate the distribution of this transition metal. □

Methods

Purification and characterization of human PrP23–98. To express the N terminus of human PrP (ref. 22), genomic DNA was amplified using primers 5'-GCCTGCGCAAGAAGCGCCCGAA-3' (encompassing the first two codons

of mature PrP and including an artificial *Fsp*I site) and 5'-CGAGTCGAC TCGGCTTGTTTCACTGACTGTG-3' (which mutates codon 99 TGG to TGA and includes a *Sal*I site). After digestion with these two enzymes the resulting fragment was ligated to pGEX cut with *Xma*I and filled in, and also cut with *Sal*I. After ligation and transformation into *E. coli* DH5a, in-frame clones were verified by DNA sequencing. After growth in the presence of IPTG, the GST/PrP23–98 fusion protein was purified by standard procedures. Approximately 1 mg fusion protein was incubated with 1 U thrombin (Sigma) for 20 min. Glutathione S-transferase (GST) and uncleaved fusion protein were removed by adding 10 ml glutathione-Sepharose 4B (Pharmacia) to the cleavage reaction. The sample was rocked at 4 °C for 30 min then centrifuged at 4,000 r.p.m. for 10 min. The supernatant was passed through a 10K cut-off CentriPlus concentrator (Amicon) and the purified HuPr23–98 was collected in the lower tube. The authenticity of PrP23–98 was verified by mass spectrometry and by using two PrP-directed antisera (not shown).

Equilibrium dialysis. This was performed using a commercial apparatus and 1K MWCO membrane (Spectrum) with protein solutions prepared in 25 mM N-ethylmorpholine buffer (pH 7.4), 150 mM KCl. Cu-chelates were made by mixing CuSO₄ solution with a twofold molar excess of glycine and adjusting the pH to 7.4. Concentrations were verified versus a Cu(II) standard (Perkin-Elmer). Protein concentrations were determined by Lowry's method and by using bicinchoninic acid (BCA) using BSA standards (Pierce). Data shown are arithmetic average values. Protein-metal mixes prepared in a variety of ratios were introduced into one compartment of a series of divided dialysis chambers, with the second compartment occupied by buffer plus salt. After equilibration for 108 h at 4 °C, metal content was determined by atomic absorption spectroscopy. Concentration of bound Cu(II) in the protein-containing chamber was deduced by subtraction of the Cu(II) content in the second chamber. Nonspecific Cu(II) binding to membranes was always <15% and typically 2–6%.

Animals and cell culture. Preparation of cerebellar cells from mice 6 days old was as described²³. The mice used were *Prnp*^{0/0} (refs 9, 24) and the appropriate controls (C57Blx129/sv or 129/Ola).

Isolation of membrane and subcellular fractions. Membrane-rich extracts were prepared from mice 5 months old by sonication in PBS. After sonication, extracts were centrifuged at 14,000 r.p.m. Material was washed with distilled water before pelleting. Isolation of subcellular fractions used a percoll gradient and was prepared from 50 ten-day-old mouse brains. Microsomal, synaptosomal fractions and endosome-enriched fractions were prepared^{25,26}. One day after plating, cerebellar cell cultures²³ were treated with 0.2 U ml⁻¹ PIPLC (Sigma) for 3 h. Cells were then collected, sonicated and the membrane fractions pelleted.

TXRF analysis. Pelleted subcellular fractions and membranes were digested by high pressure ashing after adding Ga as an internal standard. Analysis for divalent ion content was performed by TXRF²⁷. The TXRF device (Seifert) containing a molybdenum fine-focus X-ray tube was operated at 50 kV and 10–38 mA. Spectra were recorded by a Si(Li)-detector and analysed using a QX-2000 analyser (Oxford Instruments). Mass fractions were calculated on the basis of known sensitivities related to the standard.

Atomic absorption spectroscopy. Brain tissue and pelleted membrane preparations were dried at 150 °C overnight and ashed at low temperature for 24 h using the plasma processor TePla 100-E (Technics Plasma). The residue was absorbed in nitric acid. Cu measurements were performed with a Zeeman 3030 (Perkin Elmer) flameless atomic absorption spectrophotometer at a wavelength of 325.2 nm after rapid atomization at 2,000 °C in a graphite-tube cuvette HGA-70 (Perkin Elmer).

Electrophysiology. Sagittal 150 μm cerebellar slices were prepared from mice 9–12 days old and kept at 34 °C in oxygenated saline for 1–4 h. Whole-cell patch-clamp recordings were performed using an EPC-9 amplifier (HEKA). Purkinje cells were visualized directly through a $\times 63$ water-immersion objective in an upright microscope (Zeiss Axioscope). The standard internal solution contained (in mM) 140 CsCl, 10 HEPES, 10 EGTA, 1 CaCl₂, 2 MgCl₂, 4 Na₂ATP and 0.4 Na₂GTP (pH 7.3, adjusted with CsOH). The experimental chamber was continuously perfused with a saline containing 125 NaCl, 2.5 KCl 1.25 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, 1 MgCl₂, 25 glucose, 0.01 CNQX, which was bubbled continuously with a mixture of 95% O₂, 5% CO₂. All experiments were performed at room temperature (22–24 °C).

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A crucial role for B cells in neuroinvasive scrapie

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Although prion proteins are most efficiently propagated through intracerebral inoculation, peripheral administration has caused the diseases kuru, iatrogenic Creutzfeldt–Jakob disease (CJD), bovine spongiform encephalopathy (BSE) and new-variant CJD^{1,2}. The development of neurological disease after peripheral inocu-

lation depends on prion expansion within cells of the lymphoreticular system^{3,4}. Here we investigate the identity of these cells by using a panel of immune-deficient mice inoculated with prions intraperitoneally: we found that defects affecting only T lymphocytes had no apparent effect, but that all mutations that disrupted the differentiation and response of B lymphocytes prevented the development of clinical scrapie. As an absence of B cells and of antibodies correlates with severe defects in follicular dendritic cells, a lack of any of these three components may prevent the development of clinical scrapie. However, we found that scrapie developed after peripheral inoculation in mice expressing immunoglobulins that were exclusively of the M subclass and without detectable specificity for the normal form of the prion PrP^C, and in mice which had differentiated B cells but no functional follicular dendritic cells. We conclude that differentiated B cells are crucial for neuroinvasion by scrapie, regardless of the specificity of their receptors.

The effect of combined immune defects on the pathogenesis of scrapie was studied in mice deficient in Rag-2 (*rag-2*^{0/0}) (ref. 5) and Rag-1 (*rag-1*^{0/0}) (ref. 6), which lack B and T cells, in SCID (severe combined immune deficient) mice, and in Agr-deficient (*agr*^{0/0}) mice (M.S., unpublished results), which lack Rag-2 as well as the receptors for interferon- α/β and interferon- γ ⁸. For controls, we inoculated inbred mice of strains C57BL/6 and 129Sv, which are the genetic backgrounds of all other mouse strains used. To investigate the role of T cells, we used mice with targeted disruption of the genes encoding CD4 (ref. 9), CD8 (ref. 10), β_2 -microglobulin¹¹ or perforin¹². Selective ablation of B lymphocytes was studied in μ MT mice¹³, which have a targeted disruption of the transmembrane exon of the immunoglobulin μ -chain gene, do not produce any immunoglobulins and suffer from a B-cell differentiation block at the large-to-small pre-B-cell transition, yet bear complete and functional T-cell subsets.

After intracerebral (i.c.) challenge with scrapie prions, all immune-deficient mice developed clinical symptoms of scrapie. This was confirmed by histopathological analysis (not shown) and by transmission of disease to indicator *tga20* mice, which overexpress the normal prion protein (PrP^C) and are hypersensitive to scrapie¹⁴ (Table 1). Transmission to *Prnp*^{0/0} mice¹⁵, which do not express PrP^C and are resistant to scrapie¹⁶ ($n = 4$), did not induce disease after >240 days, as expected for bona fide scrapie. In all groups, latency times from inoculation to first appearance of clinical symptoms and to terminal disease (Table 2), as well as brain prion infectivity titres (Table 1), were similar to those of control mice. Thus, if prions were delivered to the central nervous system, scrapie pathogenesis and prion expansion in the brain proceeded without any detectable influence of the immune status of the host.

When mice were exposed to prions through the intraperitoneal (i.p.) route, mice with homozygous ablations of CD4, CD8, β_2 -microglobulin or perforin developed the initial symptoms of disease and terminal scrapie with latency periods similar to those of 129Sv and C57BL/6 mice (Table 2), and reached analogous prion titres in both spleen and brain (Table 1). We conclude that CD8⁺ cytotoxic and CD4⁺ helper T lymphocytes are not rate-limiting for scrapie after peripheral inoculation of prions, in agreement with the observation that nude mice develop scrapie normally after i.p. inoculation³.

In contrast, no disease appeared after i.p. inoculation in μ MT and in Rag-deficient (*rag-1*^{0/0}, *rag-2*^{0/0} and *agr*^{0/0}) mice, and no prion infectivity was detectable in their spleens (Table 1). In SCID C57BL/6 mice, disease was marginally prolonged, which disagrees with earlier results^{4,17} and may be due to incomplete immune deficiency of SCID mice in specific genetic backgrounds^{18,19}, because SCID C.B-17 mice (whose immune defect is less leaky) did not develop disease (Table 2).

Histopathological examination of brain sections revealed generalized spongiform encephalopathy in all wild-type and immune-

deficient mice clinically diagnosed as scrapie-sick (Fig. 1). In addition, and despite lack of clinical symptoms, spongiform encephalopathy was seen in 1/7 Rag-deficient and 1/6 μ MT mice (at random sampling) 342 and 436 days after i.p. inoculation (Fig. 1), and significant prion titres were found in brains of 3/7 Rag-deficient mice and 1/3 μ MT mice (Table 1). Western blot analysis revealed accumulation of the pathological form of the prion protein, PrP^{Sc}, in the brains of 2/6 Rag-deficient and 2/6 μ MT mice inoculated i.p.

(Fig. 2). The remaining Rag-deficient and μ MT mice did not accumulate PrP^{Sc} as late as 504 days after inoculation.

The latter findings are compatible with incipient scrapie in a fraction of B-cell-deficient mice. Therefore, although it prevents 'neuroinvasion' of the scrapie agent in most cases, absence of B cells uncovers a slower, <50% efficient mechanism of pathogenesis which may cause scrapie in situations of immune deficiency. Even then, B-cell deficiency prolongs the delay between PrP^{Sc} accumulation,

Table 1 Scrapie symptoms, scrapie histopathology and infectivity titres in individual immunodeficient mice

Genotype	Primary infection (route of inoculation: i.c.)			Infectivity bioassay	
	Incubation (d)	Symptoms	Pathology	Brain	Spleen
CD4 ^{0/0}	176	+	+	7.2* (65 d $\pm$ 2†)	6.6 (71 d $\pm$ 2)
CD4 ^{0/0}	154	+	+	7.4 (63 d $\pm$ 1)	7 (67 d $\pm$ 2)
SCID	167	+	+	7.3 (64 d $\pm$ 1)	5.5 (81 d $\pm$ 2)
SCID	167	+	+	7.6 (62 d $\pm$ 2)	5.2 (83 d $\pm$ 1)
<i>rag-2</i> ^{0/0}	171	+	+	7.3 (64.5 d $\pm$ 2)	<0 (>200 d)
<i>agr</i> ^{0/0}	182	+	+	7.3 (64.5 d $\pm$ 1)	<0 (>145 d)
μ MT	175	+	+	7.9 (59 d $\pm$ 5)	<0 (>200 d)
Genotype	Primary infection (route of inoculation: i.p.)			Infectivity bioassay	
	Incubation (d)	Symptoms	Pathology	Brain	Spleen
CD4 ^{0/0}	191	+	+	7.3 (64 d $\pm$ 1†)	ND
CD4 ^{0/0}	195	+	+	7.5 (62.5 d $\pm$ 2)	ND
SCID	214	+	+	7.3 (64 d $\pm$ 1)	5.2 (83 d $\pm$ 1)
SCID	249	+	+	7.7 (61 d $\pm$ 2)	5 (85 d $\pm$ 1)
<i>rag-2</i> ^{0/0}	286	–	–	6.5 (72 d $\pm$ 2)	<0 (>200 d)
<i>rag-2</i> ^{0/0}	286	–	–	<0 (>200 d)	<0 (>200 d)
<i>rag-2</i> ^{0/0}	339	–	–	<1 (>122 d)	<2 (>115 d)
<i>rag-2</i> ^{0/0}	342	–	+	7.5 (65 d $\pm$ 1)	<2 (>115 d)
<i>rag-1</i> ^{0/0}	222	–	–	<1 (>122 d)	<2 (>115 d)
<i>agr</i> ^{0/0}	284	–	+	7.2 (65 d $\pm$ 0)	<0 (>139 d)
<i>agr</i> ^{0/0}	349	–	–	<0 (>139 d)	<0 (>139 d)
μ MT	286	–	–	<0 (>200 d)	<0 (>200 d)
μ MT	286	–	–	<0 (>200 d)	<0 (>200 d)
μ MT	375	–	–	7.8 (60 d $\pm$ 1)	<0 (>200 d)
μ MT	436	–	+	ND	ND
TNFR1 ^{0/0}	211	+	+	7.7 (61 d $\pm$ 1)	ND
TNFR1 ^{0/0}	212	+	+	7.7 (60 d $\pm$ 1)	ND

For infectivity bioassays, brain or spleen homogenates were injected i.c. into groups of four *tga20* mice. ND, not determined.

* Prion titres expressed as log LD₅₀ per g of spleen or brain tissue.

† Incubation time, in days, of indicator *tga20* mice (average $\pm$ standard deviation).

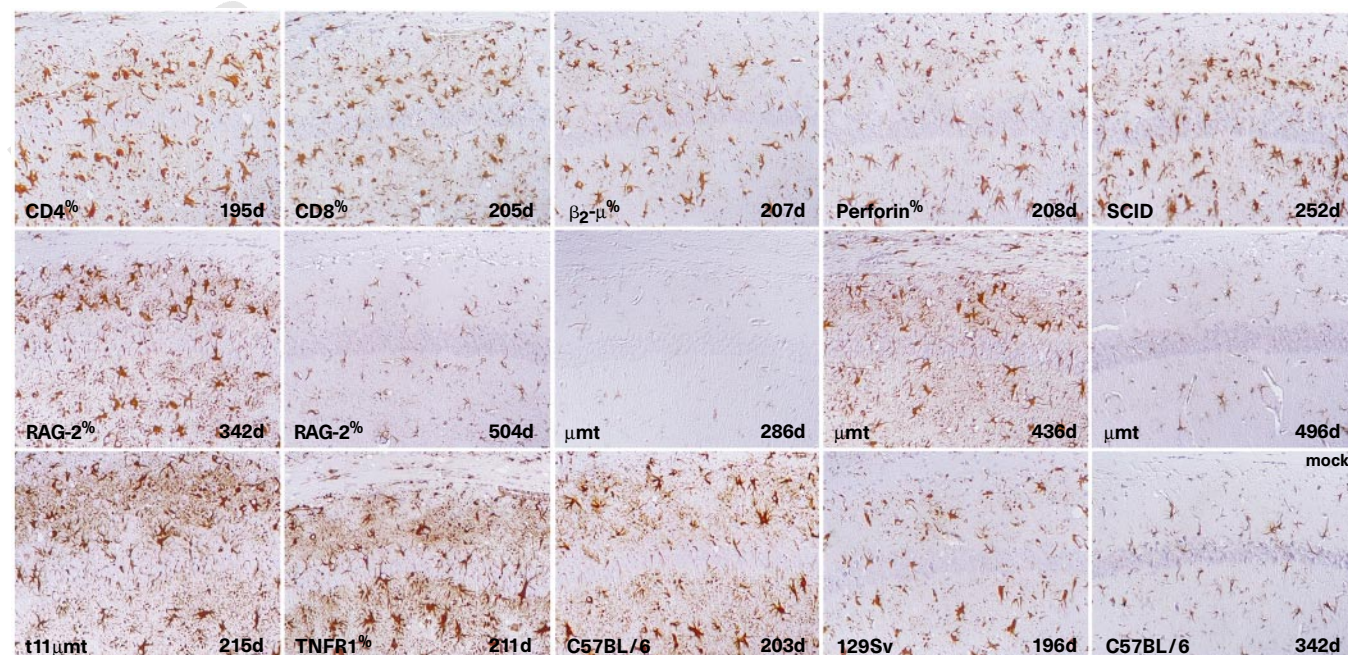


Figure 1 Brain histopathology of immune deficient and control mice after i.p. inoculation of scrapie prions. The hippocampal formation was immunostained for glial fibrillary acidic protein, and identical segments of the pyramidal cell ribbon were microphotographed (200 $\times$). Intense, diffuse gliosis was visible in brains of T-

cell-deficient, SCID, TNFR1^{0/0}, *t11* μ MT, and infected control mice. Some *rag-2*^{0/0} and μ MT mice showed spongiform encephalopathy, but others of the same genotype did not display any pathology after similar time periods following i.p. inoculation, and were indistinguishable from mock-infected C57BL/6 mice.

onset of scrapie histopathology and clinical symptoms, arguably beyond the typical life expectancy of mice.

These results suggest that B cells may 'transport' prions from lymphoid organs to nervous tissue. Alternatively, the apparent protection of B-cell-deficient mice from prions administered i.p. may result from the absence of immunoglobulins. Complexing of PrP^{Sc} with antibodies may favour nucleation (a process proposed to underlie the formation of prion infectivity²⁰) or may opsonize PrP^{Sc} and enhance access to lymphoid sites of prion expansion. To clarify

this question, we inoculated *t11μMT* mice (*μMT* mice expressing a rearranged IgM transgene directed against the glycoprotein of vesicular stomatitis virus) and found that they could support normal B-cell differentiation (U.K., unpublished) but exclusively expressed the transgenic IgM heavy chain, had a heavily skewed and very limited antibody repertoire, and lacked immunoglobulins of the D, G, E and A subclasses.

After i.p. inoculation with prions, *t11μMT* mice developed disease with a latency comparable to that of wild-type mice (Table 2)

Table 2 Latency of scrapie in different immunodeficient mice

Defect	Genotype	Intracerebral route		Intraperitoneal route	
		Scrapie	Time to terminal disease (d)	Scrapie	Time to terminal disease (d)
T	CD4 ^{0/0} *	7/7	159 ± 11	8/8	191 ± 1
T	CD8 ^{0/0} *	6/6	157 ± 15	6/6	202 ± 5
T	β ₂ -μ ^{0/0} *	8/8	162 ± 11	7/7	211 ± 6
T	Perforin ^{0/0} *	3/4†	171 ± 2	4/4	204 ± 3
T and B	SCID *	7/8‡	160 ± 11	6/8‡	226 ± 15
T and B	SCID§	4/4	152 ± 1	1/4	289
T and B	rag-2 ^{0/0} *	7/7	167 ± 2	0/7	Healthy (>504)
T and B	rag-1 ^{0/0} *	3/3	175 ± 2	0/5	Healthy (>258)
T and B	agr ^{0/0} ¶	6/6	184 ± 10	0/7	Healthy (>450)
B	μMT*	8/8	181 ± 6	0/8	Healthy (>534)
IgG	<i>t11μMT</i> *	5/5	170 ± 3	4/4	223 ± 2
FDC	TNFR1 ^{0/0} #	7/7	165 ± 3	9/9	216 ± 4
Controls	129Sv	4/4	167 ± 9	4/4	193 ± 3
	C57BL/6	4/4	166 ± 2	4/4	206 ± 2

All mice developed spongiform encephalopathy after i.c. inoculation. In contrast, B-cell-deficient mice stayed healthy after i.p. inoculation of RML scrapie prions.

* Genetic background was inbred C57BL/6.

† One *Perforin*^{0/0} and one SCID mouse suffered from intercurrent death 135 and 141 days after inoculation, respectively.

‡ Two SCID C57BL/6 mice remained healthy and were killed 303 and 323 days after inoculation.

§ Genetic background was inbred C.B-17.

|| 3/4 SCID C.B-17 mice remained healthy (>340 d). Four further SCID C.B-17 mice were challenged with 100 μl of a 10⁻⁴ dilution of RML prions, and all remained healthy (>340 d).

¶ Genetic background was C57BL/6 × 129Sv.

Genetic background was inbred 129Sv.

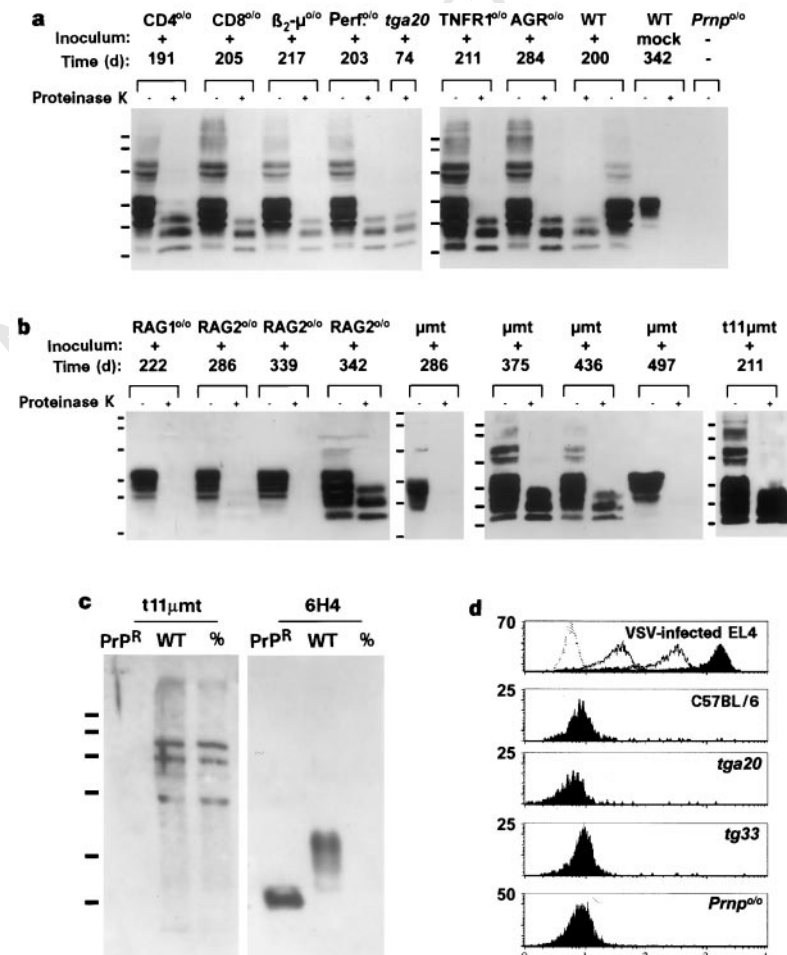


Figure 2 Western blot analysis of brains of immune-deficient mice after i.p. inoculation with scrapie prions and lack of specific antibodies against PrP in *t11μMT* mice. **a, b**, Western blots of brain material electrophoresed native (-) or after digestion with proteinase K (+). Large amounts of PK-resistant prion protein (PrP^{Sc}) were detected in all mice that had developed scrapie, as well as in one *agr*^{0/0} (**a**) two *rag-2*^{0/0} and two *μMT* mice (**b**). One further B-cell-deficient mouse proved negative for PrP^{Sc} (not shown), and no clinical symptoms of scrapie were detected in any B-cell-deficient mice irrespective of accumulation of PrP^{Sc}. **c**, Western blot prepared with recombinant murine PrP from *E. coli* (PrP^R), total brain protein extract from a wild-type mouse (WT), and total brain protein extract from a *Prnp*^{0/0} mouse (0/0)¹⁵. Blots were incubated with serum from a *t11μMT* mouse inoculated with prions i.p. (left), stripped and reprobed with monoclonal antibody 6H4 to recombinant PrP (right). The presence of PrP-specific antibodies, as indicated by a 20K band in lane PrP^R and by a cluster of bands present in lane WT but absent from lane 0/0, is evidence with 6H4 antibody but undetectable in *t11μMT* serum. Relative molecular mass markers (top to bottom): 105K, 82K, 45K, 37.3K, 28.6K, 19.4K. **d**, FACS analysis of immunoreactivity of *t11μMT* serum. Ordinate: cell counts; abscissa: logarithm of fluorescence intensity. Serum from a *t11μMT* mouse 210 days after i.p. inoculation with prions was diluted 1:10 and 1:100, stained VSV-infected EL4 cells (top panel, unfilled area) almost as strongly as VSV-specific monoclonal antibody VI24 (filled area). In contrast, immunoreaction of *t11μMT* serum (1:10) with CD3⁺ T cells from C57BL/6, *tga20*, *tg33* (ref. 29) and *Prnp*^{0/0} mice (lower panels) did not exceed background, like normal C57BL/6 serum on EL4 cells (top panel, dotted line). The same profiles were obtained when probes were stained with serum of untreated *t11μMT* mice (data not shown).

and accumulated PrP^{Sc} in their brains (Fig. 2b). Serum from both uninfected and terminally scrapie-sick *t11 μ MT* mice inoculated i.p. was shown by western blotting and by fluorescence-activated cell sorting (FACS) analysis not to crossreact with PrP^C (Fig. 2c, d), suggesting that IgGs are not the effectors of prion 'neuroinvasion', and that a specific humoral immune response (at least as assessed by FACS and western-blot analysis) cannot be correlated with peripheral pathogenesis of scrapie. However, we cannot exclude the possibility that IgMs below the threshold of detectability, or indirect effects of antibodies, may be involved in scrapie pathogenesis.

B cells are required for maturation of follicular dendritic cells (FDCs) and formation of germinal centres. Protection of B-cell-deficient mice may therefore result from the absence of FDCs, especially as FDCs accumulate PrP^{Sc} extensively in i.p.-inoculated mice³ and in the tonsils of patients suffering from new-variant CJD²¹. We inoculated mice lacking tumour-necrosis factor receptor-1 (TNFR1^{0/0})²², which have virtually no germinal centres in lymphatic organs and very few, if any, FDCs²³, despite differentiation of functional B and T lymphocytes. These mice developed scrapie after both i.c. and i.p. inoculation, as did control mice (Table 2), thus disproving a prime role for FDCs in peripheral pathogenesis and supporting our previous finding that adoptive transfer of fetal liver cells (which does not efficiently replace FDCs²⁴) can restore high spleen prion titres after i.p. inoculation²⁵.

We have identified B cells (or B-cell-dependent processes) as a limiting factor in the development of scrapie after peripheral inoculation. The suspicion that B cells may be the physical carriers of prions may eventually call for a re-evaluation of the safety of blood products. The greatly increased time between peripheral inoculation and detection of prions in the central nervous system, as well as the complete suppression of clinical symptoms, suggest that suppression of B cells could mitigate the course of spongiform encephalopathies. □

Methods

Scrapie inoculation. Mice were inoculated with a 1% homogenate of heat- and sarcosyl-treated brain prepared from mice infected with the Rocky Mountain laboratory (RML) scrapie strain. 30 μ l were used for i.c. injection, whereas 100 μ l were administered i.p. Mice were monitored every second day, and scrapie was diagnosed according to standard clinical criteria.

Western blot analysis. 10% brain homogenates were prepared as described¹⁶ and, where indicated, digested with 20 μ g ml⁻¹ proteinase K for 30 min at 37 °C. 80 μ g of total protein were then electrophoresed through 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, probed with monoclonal antibody 6H4 (a gift from B. Oesch, Prionics AG, Zurich) or polyclonal antiserum IB3 (ref. 26) against mouse PrP, and developed by enhanced chemiluminescence (Amersham).

FACS analysis. Peripheral blood cells were incubated with serum from *t11 μ MT* mice, washed, incubated with anti-mouse IgM-FITC conjugate followed by anti-CD3-PE (Pharmingen), and analysed with a Becton-Dickinson FACScan instrument after erythrocyte lysis and fixation. For analysis, cells were gated on CD3-positive T cells. EL4 cells infected with vesicular stomatitis virus (VSV) were stained with 5 μ g VSV-specific monoclonal antibody VI24 (ref. 27) and with FITC-labelled antibody to mouse IgG2a (Southern Biotechnology), or with serum of *t11 μ MT* mice, and with FITC-labelled F(ab')₂ antibody to mouse IgM (anti-IgM-FITC, Tago), or with serum of C57BL/6 mice and anti-IgM-FITC. All data acquisition and analysis were performed with CellQuest software (Becton Dickinson).

Generation of *t11 μ MT* mice. The V-gene segment of the immunoglobulin heavy chain of the immunoglobulin heavy chain of the B-cell hybridoma VI41 (ref. 27) secreting a VSV-neutralizing antibody was cloned into an expression vector encoding the mouse μ -chain of allotype a. Transgenic mice were generated and backcrossed to μ MT mice. *t11 μ MT* mice exclusively expressed the transgenic μ -chain of the allotype a; endogenous IgM of the allotype b and immunoglobulins of other subclasses were not detected in their serum (not shown).

Detection of anti-PrP antibodies. Brain lysates from wild-type and *Prnp*^{0/0} mice¹⁴, as well as recombinant *E. coli* PrP, were electrophoresed through a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. Membranes were incubated with serum from infected, terminally scrapie-sick mice (diluted 1:100). Visualization was achieved as described above.

Immunohistochemical studies. Brain tissue from each mouse was fixed, inactivated for 1 h with 98% formic acid, embedded in paraffin, and subjected to conventional staining and to immunostaining for glial fibrillary acidic protein according to standard procedures. Gliosis (a nonspecific but early indicator of brain damage) was detected by the presence of large immunostained reactive astrocytes. In terminally scrapie-sick mice, widespread vacuolation was consistently seen throughout the CNS.

Infectivity bioassays. Brain and spleen homogenates (10% in 0.32 M sucrose) were prepared from infected animals as described, and 30 μ l (diluted 1:10 in PBS and 1% BSA) were administered i.c. to groups of at least four *tga20* mice for each sample. The incubation time until development of terminal scrapie sickness was determined and infectivity titres were calculated²⁸ using the relationship $y = 14.37 - 0.11x$, where y is the ID₅₀ and x is the incubation time (in days) to terminal disease¹⁴.

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Neuregulin- β induces expression of an NMDA-receptor subunit

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Neuregulins (also known as ARIA, NDF, heregulin, GGF) are a family of widely expressed growth and differentiation factors. Neuregulins secreted from motor neurons accumulate at maturing neuromuscular junctions, where they stimulate transcription of genes encoding specific acetylcholine receptors. How these factors function at central synapses, however, is unknown. In the maturing cerebellum, neuregulins are concentrated in glutamatergic mossy fibres that innervate granule cells in the internal granule-cell layer¹. We have analysed the effects of neuregulins on the expression of genes encoding NMDA (*N*-methyl-D-aspartate) receptors in the cerebellum, because receptor composition changes dramatically as expression of the receptor NR2C subunit is specifically induced in neurons in the internal granule-cell layer during synaptogenesis. Here we report that addition of a neuregulin- β isoform to cultured cerebellar slices specifically increases the expression of NR2C messenger RNAs by at least 100-fold; effects are only minor with a neuregulin- α isoform. This stimulation of NR2C expression requires synaptic activity by NMDA receptors, as well as neuregulin- β . Addition of the NMDA-receptor-channel blocker AP-5 prevents upregulation of the NR2C subunit by neuregulin, whereas an AMPA/kainate-receptor antagonist does not. Consistent with these effects of neuregulin, we find that granule cells express its receptors ErbB2 and ErbB4 before the NR2C subunit of the NMDA receptor. Our results indicate that neuregulins regulate the composition of neurotransmitter receptors in maturing synapses in the brain, in a manner analogous to the neuromuscular junction.

NMDA receptors (NR) are neuron-specific glutamate-gated channels composed of a common NR1 subunit and distinct combinations of NR2 subunits, known as NR2A–2D (or ϵ 1– ϵ 4). The specific electrophysiological properties imparted by the NR2 subunits, and their differential regional and developmental expression, increase the diversity of NMDA-receptor function. Developmental changes are most evident in the cerebellum. Cerebellar granule cells express NR1, NR2A and NR2B mRNAs after division in the external granule-cell layer (EGL). After migration into the internal granule-cell layer (IGL), where granule cells are innervated by mossy fibres, these cells downregulate NR2B and activate NR2C expression². The differences in the electrophysiological properties of NMDA receptors in EGL and IGL neurons correlate with a switch from NR2B- to NR2C-containing receptors^{3–5}, and targeted gene-inactivation experiments confirm the contribution of NR2C-containing receptors at mossy fibre synapses^{6,7}. A possible role for mossy fibre innervation in the activation of NR2C expression was suggested by the observation that accumulation of transcripts in IGL neurons closely follows the spatial–temporal pattern of granule-cell maturation^{8,9}. As neuregulins are expressed by neurons projecting to the IGL¹, we tested whether addition of these factors to cerebellar slice cultures grown in the absence of mossy fibre inputs could stimulate expression of the NR2C gene. Slices were prepared from 9–

day-old (P9) mouse cerebella and cultured under conditions that sustain granule-cell migration and cell viability¹⁰ (see Methods). As previously shown in the rat¹⁰, migration of granule cells from the EGL to the IGL was essentially complete after 7 days *in vitro* (DIV7), when neuregulin treatment was begun (Fig. 1a, b). As shown in Fig. 2a, neuregulin- β 1 dramatically upregulates NR2C expression. In slices collected at the time neuregulin treatment was started (DIV7), or cultured in the absence of factor for DIV14, NR2C mRNA was undetectable (that is, same as background), as found previously¹¹. In contrast, addition of 5 nM neuregulin- β 1 increased NR2C mRNA expression in DIV14 slices by at least 100-fold (see later) compared with non-treated slices. Given that NR2C signals were not detectable in RNA from untreated DIV14 cultures, the size of the neuregulin response was determined in experiments in which RNA from treated cultures was serially diluted from 500 to 0.5 ng and subjected to reverse transcription followed by quantitative polymerase chain reaction (RT-PCR) (Fig. 2b). From this, we calculate that addition of neuregulin- β 1 causes a 100 to 1,000-fold increase in NR2C mRNA levels over samples from untreated slices. In contrast to NR2C, NR2B transcripts were expressed in untreated slices (DIV7 and 14) and no effect of neuregulin- β 1 on NR2B expression was observed (Fig. 2a). Likewise, neuregulin did not alter the expression of the general neuronal marker neural-specific enolase (NSE) nor did it cause changes in the amount of total RNA in the slices. The gross morphology of the slices was not affected by neuregulin treatment (Fig. 1c, d). Taken together, these results show that the effect of neuregulin- β 1 on NR2C expression is selective.

Most of the biological activity of neuregulins lies in a region encoding the EGF-like domain, although other portions of the molecule may contribute to localization¹² and function¹³. Alternative splicing generates neuregulin isoforms that differ at the C-terminal portion of the epidermal growth factor (EGF)-like domain; transcripts encoding neuregulin- β isoforms are enriched in neural tissues, whereas α -isoforms predominate in mesenchyme¹⁴. *In vitro*, recombinant proteins containing only the EGF-like domain stimulate neuregulin receptors at subnanomolar concentrations^{15,16} and activate transcription of genes encoding acetylcholine receptors (AChR)^{17–19}. To analyse the specificity of neuregulin effects on NR2C expression, slices were treated with recombinant α 2 or β 1 neuregulin isoforms made either in transfected CHO cells as 44K soluble proteins²⁰ or in *Escherichia coli* as

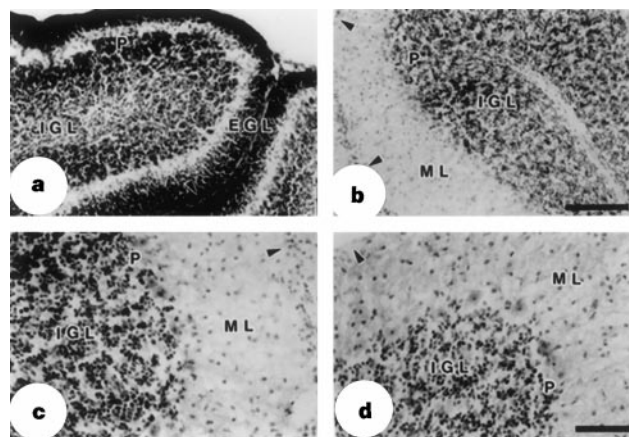


Figure 1 Cerebellar granule cells migrate in slice cultures and gross slice morphology is unaffected by neuregulin. Slices prepared from P9 mice were cultured from DIV1 (a) to DIV7 (b) in the absence of neuregulin. During the first week in culture, granule cells migrate from the EGL to the IGL. Slices were grown for an additional week either in the absence (c) or presence (d) of 5 nM neuregulin- β 1. Arrowheads mark the edge of the lobule, P indicates the Purkinje cell layer, and ML indicates the molecular layer. Scale bar in a, b is 100 μ m; in c, d, it is 50 μ m.

6.6K EGF-domain-containing peptides¹⁵. All neuregulin preparations were effective at inducing phosphorylation of the ErbB2 receptor in MDA453 human cancer cells²⁰ (results not shown). Addition of full-length $\alpha 2$ -neuregulin (at 5 nM) either had a minor effect or no effect on NR2C expression, as compared to $\beta 1$ -neuregulin (Fig. 3). Addition of either the $\beta 1$ (177–246) or $\alpha 2$ (177–241) EGF-domain proteins (at 5 nM) had no effect on NR2C expression in slice cultures (Fig. 3). These results show that other conserved domains of the protein or post-translational modifications contribute to the efficacy of neuregulin action on NR2C expression.

The action of neuregulins is mediated through ErbB receptors^{21,22}. These receptors exist as homo- and heterodimers of ErbB2, ErbB3

and ErbB4 proteins²³, which are tyrosine kinases related to the EGF receptor. As the distribution of ErbB receptors in the brain is unknown, we analysed their expression pattern in rat cerebellum using *in situ* hybridization and western blotting (Fig. 4). Hybridization of P14 cerebellum sections with specific antisense RNA probes showed that ErbB2 messenger RNAs are widely expressed by cells present in the EGL, molecular layer, IGL and the white matter.

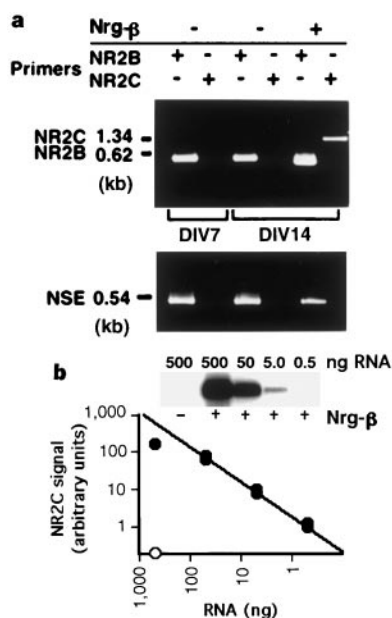


Figure 2 Neuregulin- β selectively stimulates expression of the NR2C gene. **a**, Representative reverse transcription polymerase chain reaction (RT-PCR) with assays of cerebellar slice RNA before treatment with neuregulin (DIV7) and after culturing slices for an additional 7 days (DIV14) in the absence (–) or presence (+) of full-length neuregulin- $\beta 1$. Specific PCR primers were used to amplify NR2B, NR2C and NSE. NSE serves as a control for RNA integrity and neuronal viability. These results are representative of 6 independent experiments. **b**, Relative quantification of RT-PCR products from neuregulin-treated (filled circles) or untreated (circle) slices using serial dilution of total RNA is shown on a log scale.

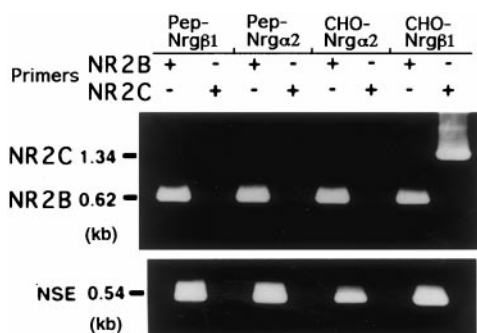


Figure 3 Neuregulin EGF-domain-containing peptides are unable to stimulate NR2C expression. Representative RT-PCR analysis of slice cultures treated either with the 6.6K *E. coli*-derived peptides Nrg $\beta 1$ (Pep-Nrg $\beta 1$; amino acids 177–246) or Nrg $\alpha 2$ (Pep-Nrg $\alpha 2$; 177–241) containing the EGF domain, or with the 44K CHO-derived Nrg $\alpha 2$ or Nrg $\beta 1$ isoforms. The final concentration of neuregulin in the cultures was 5 nM. These experiments were repeated 3 times.

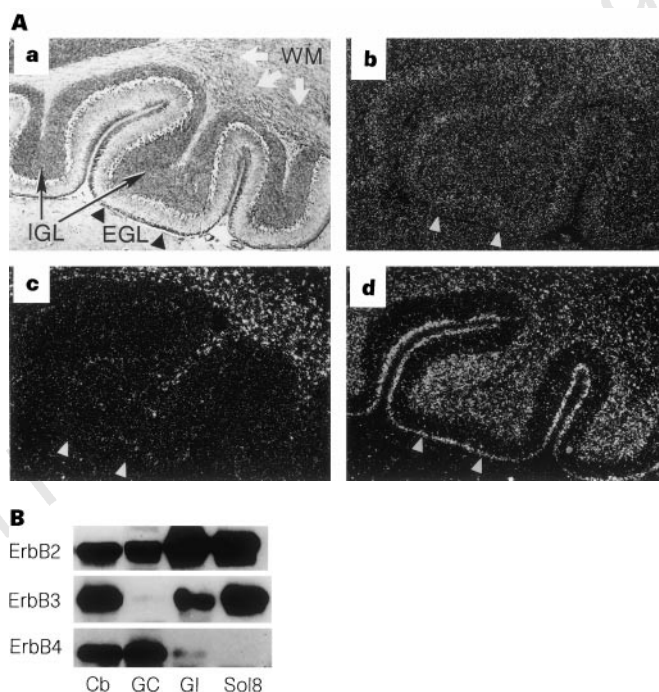


Figure 4 Neuregulin receptor mRNAs and proteins are expressed in cerebellar granule cells. **A**, *In situ* hybridization of ErbB2 (**b**), ErbB3 (**c**) and ErbB4 (**d**) transcripts on serial parasagittal sections from P14 rat cerebellum. Structures are indicated in **a**: white matter (white arrows), IGL (black arrows) and EGL (arrowheads) are indicated. Magnification, $\times 100$. **B**, Cellular distribution of ErbB proteins analysed on western blots. Whereas ErbB2–4 are found in P14 cerebellar membranes (Cb), differential *erbB* expression is seen in protein extracts made from purified cerebellar granule cells (GC) or glial cells (GI); Sol8 muscle (Sol 8) cells serve as a control.

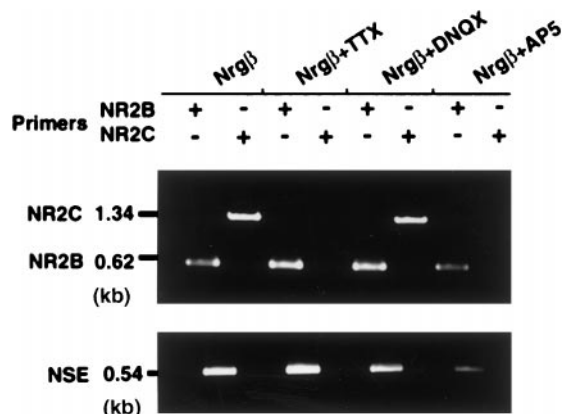


Figure 5 Neuregulin-dependent NR2C expression requires neuronal activity. Slices cultured from DIV7 to DIV14 in the presence of neuregulin- β plus the sodium-channel blocker TTX (1 μ M) failed to express NR2C. The AMPA/kainate-receptor antagonist DNQX had no effect, whereas the NMDA-receptor antagonist AP-5 blocked neuregulin-dependent NR2C expression. Experiments were done three times.

contrast, ErbB3 levels are highest in regions rich in oligodendrocytes (the myelinated tracks in the white matter), whereas ErbB4 transcripts are enriched in granule cells located in the EGL and in the IGL; a lower ErbB4 signal was detected in the white matter at this age (Fig. 4A). Using previously characterized ErbB-specific antisera^{17–19,24} on western blots, we found that the three ErbB proteins are expressed in P14 cerebellum (Fig. 4B). To determine the cellular distribution of the receptors, we analysed protein lysates made from dissociated cultures of granule cells and glia. As shown in Fig. 4B, granule-cell-enriched cultures (>95% pure) predominantly expressed the ErbB2 and ErbB4 receptors, whereas glia cell cultures expressed ErbB2 and ErbB3. These results show that granule cells express ErbB receptors early in development and strongly support the idea that neuregulins may signal through ErbB2/ErbB4 and ErbB4/ErbB4 dimers to activate expression of the NR2C gene in cerebellar granule cells.

In agreement with our results, others¹¹ have failed to detect NR2C transcripts in cerebellar slice cultures but have found that spontaneous activity in the slice could alter expression of other NMDA receptor subunits. Depolarization of cerebellar slices with KCl or veratridine causes the release of glutamate, which is blocked by the sodium-channel blocker tetrodotoxin (TTX)²⁵; we therefore tested whether spontaneous activity in the slice, possibly resulting from glutamate-receptor activation, can also contribute to the neuregulin effect on NR2C expression. The levels of NR2C mRNAs were analysed in slices treated with neuregulin-β1 (at 5 nM) plus specific blockers for sodium channels (TTX), AMPA/kainate receptors (DNQX) or NMDA receptors (AP-5). As shown in Fig. 5, blocking of spontaneous activity in the slice with 1 μM TTX prevented neuregulin-dependent NR2C expression. As spontaneous activity may function through the release of glutamate, we tested whether antagonists of glutamate receptors affected NR2C levels. Blocking of AMPA/kainate receptors by addition of 10 μM DNQX had no effect, whereas blocking of NMDA receptors by 40 μM AP-5 abolished neuregulin-dependent NR2C expression. Thus, stimulation of NR2C expression requires the activation of both neuregulin and NMDA receptors.

On the basis of these results, we propose that mossy fibre innervation provides the necessary signals (neuregulin and synaptic transmission via NMDA receptors) to stimulate NR2C expression during cerebellar granule cell maturation. As at the neuromuscular junction, where an exchange of the γ for the ε AChR subunit during synaptic maturation changes the channel properties of the receptor and results in channels passing less current²⁶, neuregulins may promote expression of lower-conducting NMDA receptors at mossy fibre synapses by increasing NR2C levels. The diverse distribution of neuregulins in the central nervous system suggests that this family of factors may regulate the composition of other channels and receptors in the brain during synaptic maturation. The requirement of converging signals elicited by neural factors and activity to modify neuronal properties may constitute a general regulatory mechanism.

Note added in proof: A structurally distinct isoform of the *Nrg-1* gene regulates the expression of nicotine-gated channels in neurons during chick embryogenesis (L. Role *et al.*, personal communication). □

Methods

Treatment of cerebellar slice cultures. Cerebellar slices from P9 mice were cultured essentially as described¹⁰. Slices were grown on 30-mm collagen-coated porous polycarbonate membranes (Millipore). The medium used has been described (15% horse serum, 25% Earle's balanced salt solution, 60% Eagle's basal medium, 5.6 g l⁻¹ glucose, 3 mM L-glutamine, 5 μg ml⁻¹ human transferrin, 5 μg ml⁻¹ bovine insulin, 30 nM sodium selenite, 20 nM progesterone, 1 mM sodium pyruvate), except that glycine (3 μM) and KCl (to 20 mM final concentration) were added to optimize migration²⁷; these modifications were necessary to observe the neuregulin effects (not shown). Cultures were

incubated in 5% CO₂/95% air at 33 °C and half-medium changes were made every other day for a week. Neuregulins were then added at 5 nM and half-medium changes containing neuregulin were made every other day for an additional 7 days. Granule-cell migration in the slice was analysed by fixing and staining 10-μm sections from the middle portion of the slice with toluidine blue. The N-terminal domain of the neuregulin-β1 isoform used (GenBank M94166) corresponds to the ARIA/NDF/HRG form²¹. The different neuregulin isoforms tested were produced in transfected CHO cells as 44K soluble proteins²⁰ or 6.6K EGF-domain-containing peptides made in *E. coli*¹⁵. We have found that neuregulin-β1 can also induce NR2C expression when added to DIV12 cultures for 2 days (not shown).

RNA analysis by RT-PCR. RNA was isolated from slice cultures using the Trizol (Gibco BRL) method. First-strand synthesis was done on 1 μg RNA in a final volume of 40 μl using random primers and Superscript reverse transcriptase (Gibco BRL). 10 μl of this reaction was taken for PCR using either NR2B-, NR2C- or NSE-specific primers, which yielded products of 0.62, 1.34 and 0.54 kb, respectively. Two different sets of NR2C PCR primers were tested and gave the same results. The specificity of the reactions was confirmed by hybridizing with ³²P-labelled internal oligonucleotide probes. To ensure that the assay was in the linear range, the cycle number and amounts of RNA were varied. Relative quantification was done by hybridizing blots of RT-PCR products from serially diluted RNA with an internal oligonucleotide, then quantifying signals using the Storm phosphorimager system (Molecular Dynamics). In an attempt to detect NR2C signals in untreated slices, cycle number was increased above the linear range.

Western blot analysis. Western blots were prepared from P14 rat cerebellar membrane preparations and total protein from dissociated cells cultured for DIV7 (ref. 28). Blots were probed with anti-ErbB antibodies (Santa Cruz) specific for each of the three ErbB receptors^{17–19,24}. The Sol8 cell line was used as a positive control for ErbB2 and ErbB3 (ref. 24). Blots were visualized using an ECL chemiluminescent detection system.

In situ hybridization. Parasagittal serial sections (10 μm) from paraffin-embedded rat P14 cerebella were hybridized with specific ³⁵S-labelled antisense cRNA probes synthesized from *erbB2* (nucleotides 3,216–3,485), *erbB3* (2,968–3,498) and *erbB4* (3,101–3,651) rat cDNAs (kindly provided by S. Carroll²⁹). As negative controls, *erbB* sense transcripts or an antisense probe for a skeletal-muscle-specific gene were used; there were no distinct hybridization signals with these probes. Probes were synthesized by runoff transcription with T3 and T7 RNA polymerases using linearized templates. *In situ* hybridization and washes were done essentially as described³⁰. Sections were exposed to NTB-3 emulsion for 4 weeks at 4 °C, processed, and analysed using both light- and dark-field microscopy.

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Cyclic-nucleotide-gated channels mediate synaptic feedback by nitric oxide

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Cyclic-nucleotide-gated (CNG) channels in outer segments of vertebrate photoreceptors generate electrical signals in response to changes in cyclic GMP concentration during phototransduction¹. CNG channels also allow the influx of Ca²⁺, which is essential for photoreceptor adaptation². In cone photoreceptors, cGMP triggers an increase in membrane capacitance indicative of exocytosis, suggesting that CNG channels are also involved in synaptic function³. Here we examine whether CNG channels reside in cone terminals and whether they regulate neurotransmitter release, specifically in response to nitric oxide (NO), a retrograde transmitter that increases cGMP synthesis and potentiates synaptic transmission in the brain^{4–6}. Using intact retina, we show that endogenous NO modulates synapses between cones and horizontal cells. In experiments on isolated cones, we show directly that CNG channels occur in clusters and are indirectly activated by S-nitrosocysteine (SNC), an NO donor. Furthermore, both SNC and pCPT–cGMP, a membrane-permeant analogue of cGMP, trigger the release of transmitter from the cone terminals. The NO-induced transmitter release is suppressed by guanylate cyclase inhibitors and prevented by direct activation of CNG channels, indicating that their activation is required for NO to elicit release. These results expand our view of CNG channel

function to include the regulation of synaptic transmission and mediation of the presynaptic effects of NO.

Patch-clamp experiments were performed on acutely dissociated cones from lizard retina, used because they possess large (5–10 µm diameter) presynaptic terminals (labelled T in Fig. 1a). By varying the duration of proteolytic enzyme treatment and trituration, we observed either intact cones or cones devoid of outer segments and/or presynaptic terminals. To investigate whether CNG channels are present in the terminals we applied the whole-cell patch-clamp configuration to cones containing terminals and cones lacking terminals. All the cones selected for use in these experiments were devoid of outer segments, to eliminate their contribution to the whole-cell CNG current. To ensure that the cone terminals were intact and functional, we applied depolarizing voltage pulses to assay for the presence of a voltage-gated Ca²⁺ current, which has been previously characterized in cones with healthy terminals^{3,7,8}.

The results of these experiments are shown in Fig. 1b. All cones exhibiting a voltage-gated Ca²⁺ current also exhibited an inward current when the membrane-permeant cGMP analogue pCPT–cGMP (8-para-chlorothio-cGMP) was applied (9 of 9 cells). In contrast, none of the cones without terminals exhibited either the voltage-gated Ca²⁺ current or pCPT–cGMP-activated current (0 of 8 cells). To confirm that the pCPT–cGMP-activated current is specifically localized to the terminal, ‘whole-terminal’ recordings were obtained after isolating the terminal from the inner segment by transecting the axon with a glass probe. Isolated terminals exhibited both the voltage-gated Ca²⁺ current and the pCPT–cGMP-activated current (Fig. 1c).

Steady-state current–voltage (*I*–*V*) relations show that the conductance activated by pCPT–cGMP is distinct from the voltage-gated Ca²⁺ conductance (Fig. 2). Application of pCPT–cGMP selectively activates a voltage-independent conductance with a reversal potential near 0 mV, without affecting the voltage-gated Ca²⁺ conductance. In contrast, nifedipine reduces but does not completely block the voltage-gated Ca²⁺ conductance, as reported previously⁸, but has no effect on the pCPT–cGMP-activated conductance.

To determine whether the conductance activated by pCPT–cGMP results directly from activation of CNG channels or indirectly from other effects of cGMP, such as activation of cGMP-dependent protein kinase, we applied cGMP onto excised inside-out patches from cone terminals in the absence of ATP, GTP or added kinases. A recording from a patch containing several CNG channels directly activated by cGMP is shown in Fig. 3a. Like other CNG channels¹, these channels exhibited voltage-dependent block after the addition of divalent cations (1 mM Ca²⁺ or Mg²⁺; data not shown), which accounts for the fact that the currents observed in patches under divalent-free conditions were much larger than the whole-cell currents obtained with divalents present. Dose–response curves fitted with the Hill equation (Fig. 3b) showed that the CNG channels in excised patches had an unusually low sensitivity to cGMP with a *K*_{1/2} of activation of 206 ± 23 µM (*n* = 9), but up to 2 mM cAMP caused no activation of the CNG channels. CNG channels in patches excised from outer segments of these cones had a similar sensitivity, with a *K*_{1/2} for cGMP of 159 ± 29 µM (*n* = 6). Both the terminal and outer-segment CNG channels were much more sensitive to pCPT–cGMP with a *K*_{1/2} of activation of 10.3 ± 3.9 µM (*n* = 5), consistent with the higher sensitivity of other CNG channels for this analogue⁹.

To investigate further the subcellular distribution of CNG channels, excised patches were made from outer segments, inner segments, and terminals of cones (Fig. 3c). Most patches (67%) from outer segments exhibited CNG channels. Moreover, these patches always contained many channels (9–19 per patch). In contrast, patches from inner segments rarely exhibited CNG channels (14%), and those that did always contained a low density of channels (2–5 channels per patch). Hence, as has been shown in rods¹⁰, CNG

channels in cones are much more densely packed in outer segments than in inner segments. Patches from the terminals, like those from inner segments, rarely contained CNG channels (31%), but those that did contained a high density of channels (13–34 channels per patch). Taken together with the whole-cell data, these patch results suggest that CNG channels are clustered in cone terminals. CNG channels would be ideally positioned to influence neurotransmitter release if these clusters were located near release sites.

What regulates the activity of CNG channels in cone terminals? CNG channels in terminals might be constitutively activated by resting levels of cGMP, like CNG channels in rod and cone outer segments, which are kept active in the dark by cytoplasmic levels of cGMP¹. In addition, neurotransmitters released onto cone terminals from other retinal cells might alter the activity of CNG channels. Nitric oxide (NO) is a strong candidate to be a retinal transmitter that could modulate the activity of cone terminal CNG channels, for several reasons. First, NO synthase is found in rod and cone photoreceptors, and also in processes of bipolar and glial cells in the outer plexiform layer of the retina, adjacent to terminals of photoreceptors^{11,12}. Second, in rods, NO has been shown to modulate the voltage-gated Ca^{2+} conductance and a voltage-independent conductance¹². Finally, the soluble form of guanylate cyclase, which synthesizes cGMP, is activated by NO and is found predominantly in inner, rather than outer, segments of cones¹³.

To test whether NO affects synaptic transmission from cone photoreceptors, we exposed intact retinas from tiger salamanders to flashes of light, and examined the voltage response of horizontal cells, which receive input from cones (Fig. 4a, b). The hyperpolarizing light response of horizontal cells exhibits a delayed depolarizing sag that is partly dependent on feedback inhibition of cone terminals by the horizontal cells themselves^{14–16}. Incubation of retina with a competitive inhibitor of NOS, N^G -nitro-L-arginine (L-NNA), resulted in a dose-dependent suppression of the sag in the horizontal cell response, as compared with control. Incubation of retina with L-arginine, the normal substrate of NOS, had no effect on the sag response, but prevented the inhibition caused by L-NNA. Incubation with ODQ, a specific inhibitor of nitric oxide-sensitive guanylate cyclase¹⁷, also significantly reduced the sag, although to a lesser extent. These results indicate that endogenous production of NO in the retina modulates synaptic communication between cones and horizontal cells, in part by stimulating the synthesis of cGMP.

Does NO alter neurotransmitter release from cone terminals by activating CNG channels? To determine whether NO causes the activation of CNG channels, we applied S-nitrosocysteine (SNC), an NO donor, onto isolated lizard cone terminals. We found that

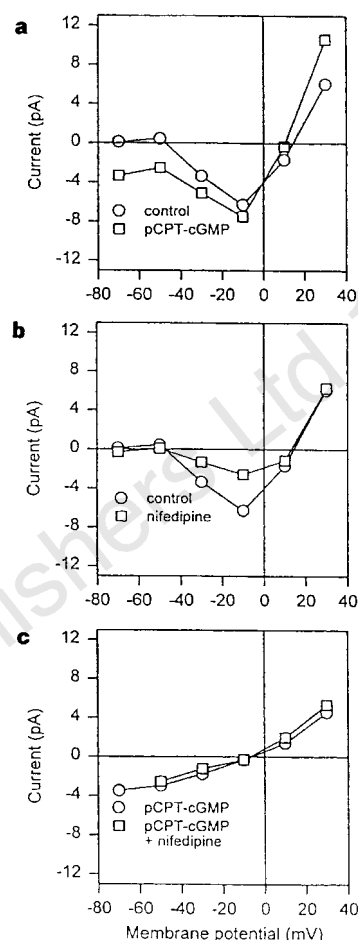
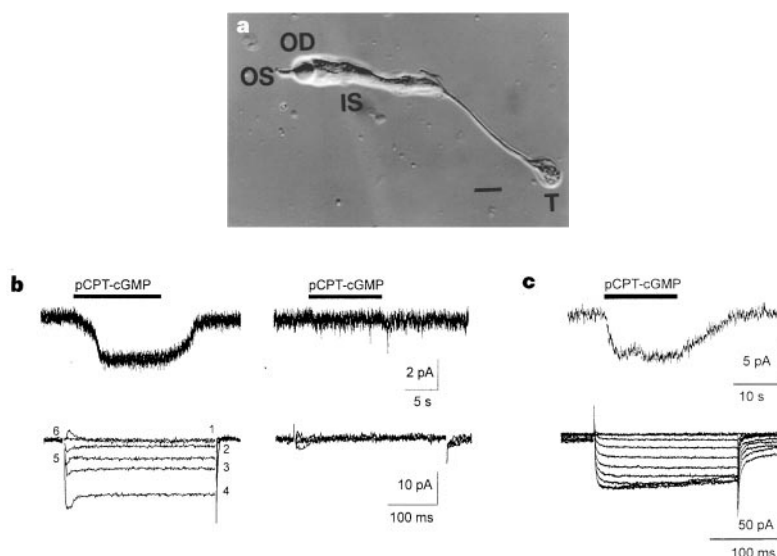


Figure 2 Steady-state I - V curves of the pCPT-cGMP-activated and voltage-gated Ca^{2+} currents. **a**, I - V curve in normal saline (control) and after application of $100 \mu\text{M}$ pCPT-cGMP. **b**, I - V curve in normal saline and after application of $1 \mu\text{M}$ nifedipine. **c**, I - V relation of the pCPT-cGMP-activated conductance obtained by subtraction of the I - V curve in control saline from the I - V curve in pCPT-cGMP, both obtained in the absence (circles) or presence (squares) of $1 \mu\text{M}$ nifedipine. All currents were measured at the end of 200-ms voltage pulses from a holding potential of -60 mV .

Figure 1 Responses of cone terminals to pCPT-cGMP. **a**, Acutely dissociated cone photoreceptor from *Anolis carolinensis* retina. Note the outer segment (OS), oil droplet (OD), inner segment (IS), and presynaptic terminal, or 'pedicle', of the cone (T). Scale bar, $10 \mu\text{m}$. **b**, Records from a cone containing a terminal (left) and from a cone lacking a terminal (right). Only the cone with the terminal exhibits an inward current in response to $100 \mu\text{M}$ pCPT-cGMP (top traces; holding potential = -60 mV). Similarly, only the cone with the terminal exhibits voltage-gated Ca^{2+} currents in response to 20 mV incremental depolarizations from -60 mV to $+40 \text{ mV}$ (bottom traces). **c**, An isolated terminal exhibits current in response to $100 \mu\text{M}$ pCPT-cGMP and 10 mV incremental depolarizations from -60 to $+30 \text{ mV}$. Tail currents result from Ca^{2+} -activated Cl^- channels^{7,31}, owing to the presence of 140 mM Cl^- in the pipette, rather than 30 mM Cl^- plus 110 mM aspartate, as in **b**.



SNC activates an inward current (Fig. 4c) associated with a voltage-insensitive conductance with a reversal potential near 0 mV (data not shown). Moreover, the SNC-elicited current is mimicked and occluded by pCPT-cGMP (Fig. 4d), suggesting that SNC and pCPT-cGMP converge to activate the same population of ion channels. Similar results were observed in a total of four experiments. Application of NO-depleted SNC solution (prepared >12 h before use) had no effect on the conductance of cones ($n = 4$).

As well as indirectly activating CNG channels by stimulating cGMP synthesis, NO directly activates CNG channels in olfactory neurons by covalently modifying the channel protein¹⁸. In our experiments, SNC applied directly onto excised inside-out patches from cone terminals failed to activate CNG channels and had no effect on the sensitivity of the channels to cGMP ($n = 5$). Therefore, it is likely that NO acts by stimulating cGMP production to activate the cone terminal CNG channels.

Does the regulation of CNG channels by NO lead to an increase in neurotransmitter release from cones? To monitor release of the cone neurotransmitter glutamate¹⁹, we used cultured horizontal cells from catfish retina as biosensors²⁰ (Fig. 5a). A dissociated cone devoid of its outer segment was manipulated so that its terminal directly contacted the horizontal cell, which was monitored with a

whole-cell patch pipette. The horizontal cells generate a detectable response to as little as 0.5 μM glutamate through the activation of NMDA and non-NMDA receptors, and little desensitization is observed during prolonged (5 s) applications of glutamate (Fig. 5b). Owing to slow changes in the holding current, responses to low glutamate concentrations (<2 μM) more reliably induced increases in the current variance ('noise') than changes in the mean current. Application of pCPT-cGMP or SNC onto the cone terminal-horizontal cell pair elicited a reversible increase in current variance (Fig. 5c, d). This increase in variance was blocked by application of 1 μM kynurenic acid, which blocks glutamate responses in horizontal cells²⁰. Moreover, although isolated horizontal cells sometimes responded to pCPT-cGMP and SNC with an outward shift in the holding current, there was no increase in noise unless the cone terminal was present. Hence the increase in the current variance results from release of glutamate from the cone terminal.

The results of these release experiments are summarized in Fig. 5d. Both pCPT-cGMP and SNC elicited three- to fourfold increases in current variance. Coapplication of pCPT-cGMP and SNC resulted in no further increase in variance, suggesting that NO triggers release through the same mechanisms as pCPT-cGMP. However,

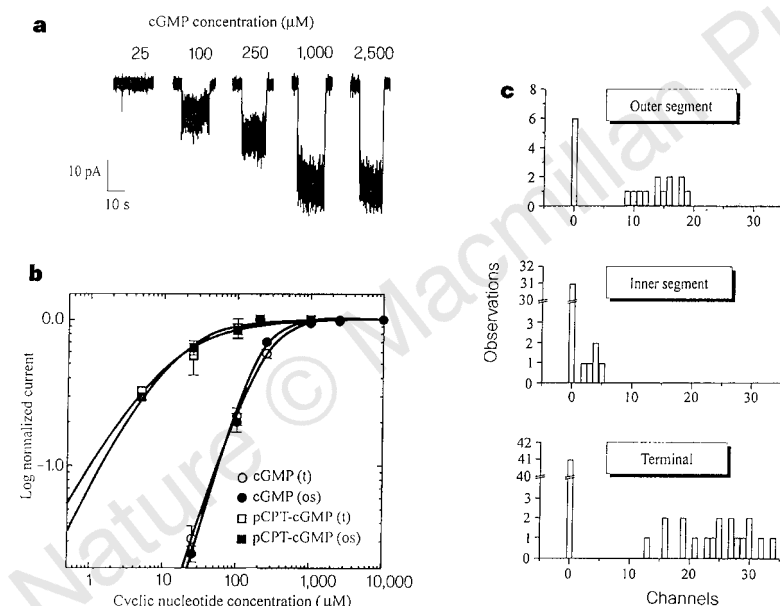


Figure 3 CNG channels in cone terminals. **a**, Direct activation of CNG channels in an inside-out patch excised from a cone terminal. The patch contained an estimated 29 channels. **b**, Dose-response curves for cone terminal (t) and outer segment (os) CNG-channel activation by cGMP and pCPT-cGMP. Continuous curves show fits to the Hill equation. **c**, Distribution of CNG channels within cones.

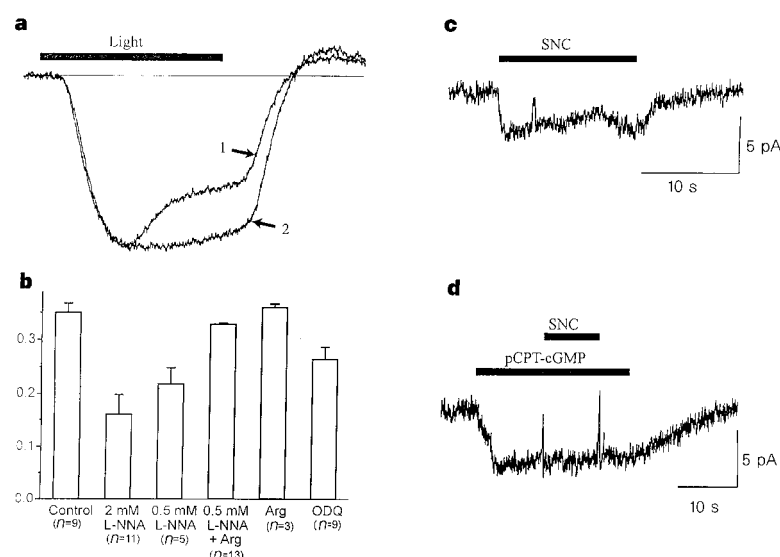


Figure 4 Modulation of cone synapses by NO. **a**, Endogenous NO modulates synaptic communication between cones and horizontal cells. Voltage responses from horizontal cells incubated in normal saline (trace 1) or in 2 mM L-NNA (trace 2) to 500-ms steps of full-field illumination ($2 \times 10^{-7} \mu\text{W} \mu\text{m}^{-2}$). Control and L-NNA responses were normalized to peak amplitudes (15 and 10 mV, respectively). **b**, Summary of feedback response experiments, showing mean $\pm$ s.e.m. of the 'response sag', defined as $1 - (S/P)$, where P is the peak voltage and S is the voltage at the end of the light step. Recordings were made from horizontal cells in retinas superfused for 1 h with control saline, L-NNA, 2 mM L-arginine, 2 mM L-arginine + L-NNA, or 100 μM ODQ, as indicated. Results with ODQ ($P < 0.02$) or L-NNA alone ($P < 0.001$) were statistically different from control. **c**, Activation of CNG current in an isolated lizard cone terminal by 50 μM SNC. **d**, Occlusion of the SNC response by prior application of 100 μM pCPT-cGMP.

application of 10 μM ODQ or 10 μM LY-83583, both inhibitors of soluble guanylate cyclase^{17,21}, blocked the action of SNC. Hence the increase in transmitter release elicited by SNC seems to be dependent upon cGMP synthesis. Because cGMP selectively activates CNG channels, these results suggest that the increase in glutamate release elicited by NO is mediated by CNG channels.

Cone terminals process two types of signals: those generated in the outer segment in response to light and those elicited by feedback transmitters released from adjacent retinal cells. Although the signal relayed from the outer segment to the terminal is crucial for rapid light responses, it is the feedback onto cone terminals that modulates the gain of the cone outputs synapse and mediates colour opponency and surround responses, and thereby underlies an important early step in the processing of visual information¹⁶. The long diffusion distance between the outer segment and the terminal makes it unlikely that CNG channels in the terminal are affected by the decrease in cGMP resulting from phototransduction. However, the activation threshold of voltage-gated Ca^{2+} channels in cones (above -45 mV ; Fig. 2 and refs 3, 7, 22) may not fully account for the voltage sensitivity of synaptic transmission from cones during light

responses, which is apparent even at -70 mV (refs 3, 23). Our results show that CNG channels are present in high-density clusters in cone terminals. These channels may be tonically activated by steady-state levels of cGMP and contribute to transmitter release by allowing Ca^{2+} influx even at hyperpolarized potentials. These CNG channels also mediate the effects of NO on neurotransmitter release from cones. NO^{5,24} and cGMP²⁵ also increase neurotransmitter release in the hippocampus, where they have been implicated in mediating long-term potentiation of synaptic transmission. The recent discovery of CNG channels in the hippocampus^{26,27} and elsewhere in the central nervous system²⁸ raises the possibility that the mechanism we have described here may be more widely used in the brain as a retrograde signalling pathway to modulate synaptic transmission. □

Methods

Electrophysiological recording from cones. Retinas from lizards (*Anolis sagrei*, collected locally, or *A. carolinensis*, from Nasco, Fort Atkinson, WI) were isolated, treated with papain, and triturated to obtain dissociated cones, as described⁷. Cells were placed in saline containing (in mM): 150 NaCl, 4 KCl, 2 CaCl_2 , 2 MgCl_2 , 5 glucose and 10 NaHEPES, pH 7.5. For whole-cell recordings, patch pipettes contained (in mM): 110 Cs aspartate, 30 CsCl, 0.5 MgCl_2 , 5 EGTA, 0.1 GTP, 0.1 ATP and 10 CsHEPES, pH 7.5, which isolated CNG and voltage-gated Ca^{2+} current from K^+ and Cl^- currents. Patch-clamp recordings were made with an Axopatch 200A (Axon Instruments) and data were analysed using p-Clamp software. All experiments were performed at $21\text{--}23^\circ\text{C}$. For excised patch experiments, both the patch pipette and the perfusion solution contained (in mM): 155 NaCl, 5 KCl, 5 EGTA and 5 NaHEPES, pH 7.5. Perfusion solutions were administered at $1\text{--}2\text{ ml min}^{-1}$. Cyclic GMP (Sigma) and 8-*p*-chlorophenylthio-guanosine 3',5'-monophosphate (pCPT-cGMP; BioLog) were prepared daily from concentrated stock solutions.

Analysis of channel density and distribution. To estimate the minimum number of active CNG channels in excised patches, the total cGMP-activated conductance was determined at saturating cGMP (2 mM) and divided by the single-channel conductance (21 pS) determined in separate experiments. Single-channel analysis showed that the maximal open probability of the cone CNG channels was high ($>90\%$), indicating that the ratio of total conductance to single-channel conductance not only indicates the minimum number of channels, but is a good estimate of the absolute channel number²⁹. In the analysis of channel distribution in cones, open excised patches were distinguished from resealed membrane vesicles by examining current noise and patch resistance in the absence of cGMP. In 205 patches from all regions of the cone there was a bimodal distribution of these parameters, with patches in one group exhibiting root mean squared noise levels $<0.25\text{ pA}$ and resistances $>2.5\text{ G}\Omega$. Application of cGMP never elicited channel activity in these patches. We assume that these represent recordings from resealed vesicles; they have been disregarded from further analysis.

Biosensor measurements of glutamate release. Cultures of cone-driven horizontal cells from the retina of catfish (*Ictalurus punctatus*) were prepared as described²⁰. After 1–5 days in culture, culture dishes containing the cells were transferred to the electrophysiology set-up and acutely dissociated *Anolis* cones were added. An isolated cone or a group of 2–3 cones were manipulated to bring their terminals into direct contact with the horizontal cell. Whole-cell patch-clamp recordings were obtained from the horizontal cell and $0.5\text{--}100\text{ }\mu\text{M}$ glutamate was applied. Only cells with detectable responses to $\leq 1\text{ }\mu\text{M}$ glutamate were used. The whole-cell pipette solution contained (in mM): 110 CsCl, 2 MgCl_2 , 0.5 CaCl_2 , 5 EGTA, 5 Na_2ATP and 10 NaHEPES, pH 7.5. cGMP modulates ionic currents in horizontal cells by the activation of cGMP-dependent protein kinase (PKG)³⁰. KT-5823 (10 μM ; Calbiochem), a PKG inhibitor, blocks these effects, so it was also included in the whole-cell patch solution. Horizontal cells were held at -40 mV and currents filtered at 5 kHz. Current records were analysed using Origin (Microcal) for determination of current variance. Statistical differences were evaluated using one-way analysis of variance.

Intact retina experiments. Tiger salamanders (*Ambystoma tigrinum*) maintained at 5°C were killed and pithed and the eyes removed under dim illumination. The cornea, lens and iris were removed and the resulting retinal

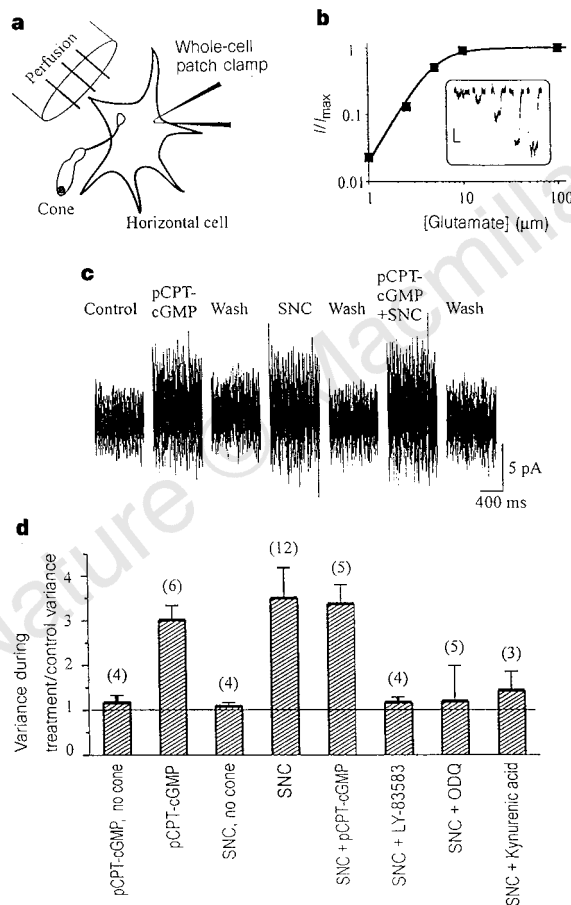


Figure 5 Biosensor measurements of transmitter release from cone terminals. **a**, Experimental arrangement. **b**, Sensitivity of the glutamate response of the horizontal cell biosensor. Bath application of 1, 2.5, 5, 10 and $100\text{ }\mu\text{M}$ glutamate elicits the responses shown in the inset (vertical scale 10 pA , horizontal scale 10 s). The graph shows the Hill fit to peak responses with a $K_{1/2}$ of $4.9\text{ }\mu\text{M}$. **c**, Steady-state changes in current noise in a horizontal cell contacted by the terminal of a dissociated cone. Recordings show reversible effects of $100\text{ }\mu\text{M}$ pCPT-cGMP and $50\text{ }\mu\text{M}$ SNC applied alone, and pCPT-cGMP and SNC applied together. **d**, Summary of release experiments. Ratio of horizontal-cell current variance during treatment to control variance. Bars show mean variance $\pm$ s.e.m., with number of cells tested in parentheses. Results for pCPT-cGMP, SNC and SNC + pCPT-cGMP were statistically different from control ($P < 0.001$).

eyecup allowed to dark-adapt for at least 1 h. Glass microelectrodes filled with 3 M K-acetate and 200 mM KCl (150–200 MΩ) were used for intracellular recording from horizontal cells. Voltage responses to light flashes were amplified with a WPI M707 Microprobe system and analysed using Basic-Fastlab software. Solutions at 21–23 °C were continuously superfused at 1 ml min⁻¹. Control saline contained (in mM): 95 NaCl, 2.5 KCl, 3 CaCl₂, 1.5 MgCl₂, 30 NaHCO₃ and 1 mg ml⁻¹ glucose, and was bubbled continuously with 95% O₂/5% CO₂ to maintain a pH of 7.6. Each recording was obtained from individual horizontal retinal cells exposed for 1 h to control saline, saline containing N^G-nitro-L-arginine (L-NNA; Calbiochem). L-arginine (Sigma) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris).

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Identification and role of adenylyl cyclase in auxin signalling in higher plants

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Cyclic AMP is an important signalling molecule in prokaryotes and eukaryotes¹, but its significance in higher plants has been generally doubted² because they have low adenylyl cyclase activity and barely detectable amounts of cAMP³. Here we used activation T-DNA tagging to create tobacco cell lines that can proliferate in the absence of the phytohormone auxin in the culture media^{4,5}. The sequence tagged in one line, *axi 141*, was used to isolate a complementary DNA encoding adenylyl cyclase, the first from a higher plant. Sequence analysis reveals that the tobacco adenylyl cyclase is probably soluble, contains characteristic leucine-rich repeats, and bears similarity with adenylyl cyclase from the yeast *Schizosaccharomyces pombe*. Expression of the cDNA in *Escherichia coli* results in an increase in endogenous cAMP levels, and in yeast its expression functionally complements the *cry1* mutation. Tobacco protoplasts treated with cAMP, or the adenylyl cyclase activator forskolin, no longer require auxin to divide. This finding, together with the observation that the adenylyl cyclase inhibitor dideoxyadenosine inhibits cell proliferation in the presence of auxin, suggests that cAMP is involved in auxin-triggered cell division in higher plants.

The molecular basis of auxin action in plants is little understood, and to address this we have developed the technique of activation T-DNA tagging^{4,5}. This involves transforming tobacco cells with a gene tag containing multiple transcriptional enhancers so that, once the tag has been inserted into the genome, expression of flanking plant DNA becomes deregulated, producing a dominant mutation. This allows direct selection for a defined phenotype, and we have selected for cells that proliferate in culture in the absence of exogenously applied auxin, with the idea that tagged genes are likely to play a role in auxin signal transduction. Genetic segregation (data not shown) and Southern analysis revealed that the auxin-independent tobacco cell line *axi 141* contains a single non-rearranged T-DNA insert (Fig. 1a, b). Plasmid rescue using *EcoRI*-digested *axi 141* genomic DNA resulted in the recovery of pAI411 containing the T-DNA flanked by approximately 3.0 kilobases (kb) and 7.5 kb of plant DNA at the right and left T-DNA borders, respectively (Fig. 1a). Transfecting tobacco protoplasts with deletion derivatives of pAI411 followed by screening for auxin-independent growth indicated that the responsible sequence was located near the left T-DNA border (Fig. 1a). The *Apal*–*Clal* fragment of pAI411 was used to screen a cDNA library from dividing tobacco protoplasts, and resulted in pB149 containing an insert of 1,600 base pairs (bp) (GenBank accession number: AF026389). Southern analysis using pB149 indicates that the sequence is present in low copy number in the tobacco genome, and revealed a restriction fragment length polymorphism between untransformed tobacco and *axi 141*, indicating that the T-DNA had inserted into an 11-kb *EcoRI* fragment of tobacco genomic DNA (Fig. 1b). Northern analysis using RNA isolated from protoplasts from untransformed and *axi 141* plants cultured in the presence or absence of auxin revealed a band of approximately 1.5 kb (Fig. 1c).

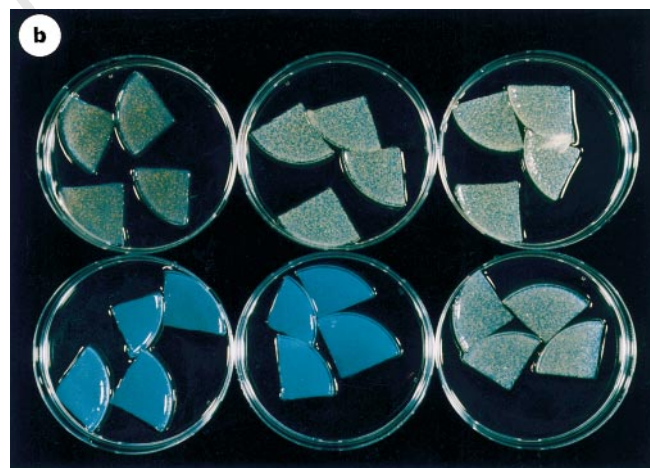
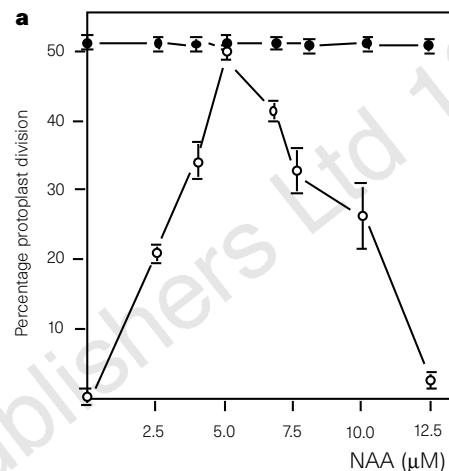
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Quantification of hybridization intensities by comparison with an actin probe revealed that *axi 141* mRNA accumulates in protoplasts from untransformed plants cultivated both in the presence and the absence of auxin. The *axi 141* protoplasts accumulate highest levels of transcript in the absence of auxin, suggesting that in the mutant line there is a feedback transcriptional regulation in the presence of auxin, as seen previously in another auxin-independent mutant *axi 1* (ref. 6).

Plants regenerated from *axi 141* cells display no obvious morphological differences compared with untransformed tobacco. However, *axi 141* protoplasts proliferate in culture not only in the absence of exogenously applied auxin but also at levels of auxin that normally inhibit division (Fig. 2a). When the cDNA insert of pB149 is cloned between the promoter and polyadenylation signals of the CaMV 35S RNA to produce pRA14-9 and used in protoplast transformation, transfected protoplasts were able to grow in the absence of exogenously applied auxin (Fig. 2b). Sequencing pB149 revealed a long open reading frame (ORF) potentially encoding a 406-amino-acid peptide, a 105-bp 5' leader sequence containing seven translational stop sites, a 160-bp 3' untranslated region, and a poly(A) tail. We have called the gene encoded by pB149 *axi 141* (for auxin independent). Analysis of the predicted protein encoded by *axi 141* shows that it contains blocks of leucine-rich repeats, and sequence comparison reveals that the carboxy terminus of the gene contains low-level similarity to the C terminus of adenylyl cyclase of *S. pombe*⁷, arrayed in regions of sequence identity ranging from 23% to 71% (Fig. 2c).

To confirm the identity of *axi 141*, the coding region of pB149 was cloned into an *E. coli* expression vector, pRSET⁸, transformed into *E. coli*, and the resulting cAMP levels in cell extracts were measured. Bacteria containing the plasmid expressing *axi 141*, checked by western analysis (data not shown), consistently accumulated 50% more cAMP than bacteria transformed with the expression vector lacking an insert (Fig. 3a). In yeast, the *CYR1* locus encodes the structural gene for adenylyl cyclase⁹. In the absence of cAMP, growth of *cyr1* mutants is arrested at the G1 phase of the cell cycle⁹. Thus, *axi 141* was cloned into a yeast expression vector and transformed

into the *cyr1* is mutant T50-3A¹⁰. The results indicate that *axi 141* functionally complements the *cyr1* mutation in a manner similar to the *cyr1* gene (Fig. 3b). Taken together, sequence similarities, raised levels of cAMP in bacteria expressing *axi 141*, and the functional complementation of the *cyr1* mutation by *axi 141*, indicate that *axi 141* probably encodes a tobacco adenylyl cyclase.



c

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1  MQRVLKARQL VRVLRKSSSP ILLNSVSRIQ SHCTYEATES CLNSSRRGY
51  FTSGTAICGN YMQTKHNIQR NVCQCVKST MLKASFSTEA GTVESSAATV
101 SVKELYDKML KSVVEQRSAP PNAWLWSLIQ SCANREDVNL LHDILQRLRI
151 FRLSNLRIHE NFNCALCQDI TKACVRVGA I DLGKKVLWKH NVYGLTPNIG
201 SAHHLLLFKA QHNDVKLLVE IMKLVKKNDL NLQPGTAEIV FSICFQTDNW
251 DLMCKYGRV VKAGVKLRKT SLDTWMEFAS KIGDVALWK IEKIRSESMK
301 EHTLASGLSC AKAFLIDHKP GDAAAIQSL NQTIPDSRRQ NFMIELQKLV
351 ADWPLEVIKR QKDEKRKELA ATLQHDIPAM LSALPNRGLN LDINLEDLTR
401 KEGVLS
    
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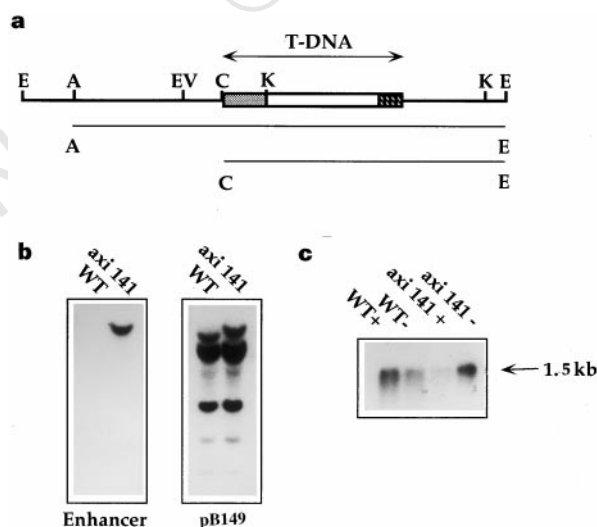


Figure 1 T-DNA organization and *axi 141* expression. **a**, T-DNA comprises a hygromycin gene (stippled box); pIC (open box); and 35S RNA enhancers (hatched boxes)⁴. Restriction sites: E, *EcoRI*; A, *Apal*; EV, *EcoRV*; C, *Clal*; and K, *KpnI*. The *Apal*-*EcoRI* fragment confers auxin-independent protoplast growth, but the *Clal*-*EcoRI* fragment does not. **b**, *EcoRI*-cut genomic DNA from tobacco (WT) and *axi 141* probed with the enhancer or pB149. **c**, Northern analysis. Total protoplast RNA from untransformed plants (WT), or *axi 141* (axi 141) cultured for 3 days with (+) or without (−) auxin probed with RNA from pB149.

Figure 2 Functional and sequence characterization of *axi 141*. **a**, Protoplasts from untransformed (open circles) and *axi 141* plants (filled circles) cultivated under differing concentrations of auxin in the presence of cytokinin. **b**, *Axi 141* triggers auxin-independent growth. Top row, protoplasts cultured in auxin, bottom row, protoplasts cultured without auxin. Left to right, untransfected protoplasts, protoplasts transfected with the cloning vector alone, and protoplasts transfected with pRA14-9. **c**, Predicted sequence of the protein encoded by pB149. Repeats containing leucine are underlined, and the region containing greatest similarity to *S. pombe* adenylyl cyclase is boxed.

The finding that deregulated expression of *axi 141* results in auxin-independent growth in isolated protoplasts led us to test whether cAMP might be involved in this. Tobacco protoplasts under defined culture conditions require both auxin and cytokinin to achieve optimal frequencies of cell division^{11,12}. Protoplasts incubated with cytokinin in the presence of the membrane-permeable dibutyl-AMP (Fig. 4a). The *axi 141* protoplasts divide in the absence of auxin and are slightly more tolerant of dibutyl-AMP than are protoplasts from untransformed plants. In contrast, in control experiments no response was seen when protoplasts were cultured with differing levels of AMP in the presence of cytokinin. There could be two ways in which cAMP replaces auxin in triggering tobacco protoplast division: cAMP is part of either the auxin signal-transduction pathway, or a separate pathway. We adopted a pharmacological approach to differentiate between these two possibilities. Auxin-induced cell division can be inhibited by pretreating protoplasts with the anti-auxin 2-NAA¹³; adenyl cyclase activity can either be inhibited by dideoxyadenosine (ddA) or stimulated by the diterpene forskolin^{14,15}. Application of 400 μ M forskolin stimulated maximal protoplast division in protoplasts from untransformed plants in the absence of auxin (Fig. 4b). Stimulation of cell division by either dibutyl-AMP or forskolin was unaffected by pretreatment of the protoplasts with 2-NAA. However, application of ddA inhibited protoplast division in the presence of auxin. This inhibition was overcome by the addition of dibutyl-AMP (Fig. 4b). These observations confirm the idea that cAMP can replace auxin in triggering cell division in tobacco protoplasts, and that this

is a result of adenyl cyclase activity. That ddA inhibits auxin-triggered cell division, but the inhibition is overcome by cAMP, suggests that cAMP is part of the auxin signal-transduction pathway.

Adenyl cyclases in general are characterized by membrane-spanning regions, leucine-rich repeats, and the conservation of sequence at the active site located towards the C terminus of the gene¹⁶. Although the structure of *axi 141* is similar to that of known adenyl cyclases, it lacks a membrane-spanning region. Size similarity of the cDNA to the signal detected on northern analysis and an inability to detect a longer transcript by 5' RACE (data not shown) lead us to suspect that pB149 is not a partial cDNA. This notion is further strengthened by the finding of a 1.4-kb *Arabidopsis* cDNA

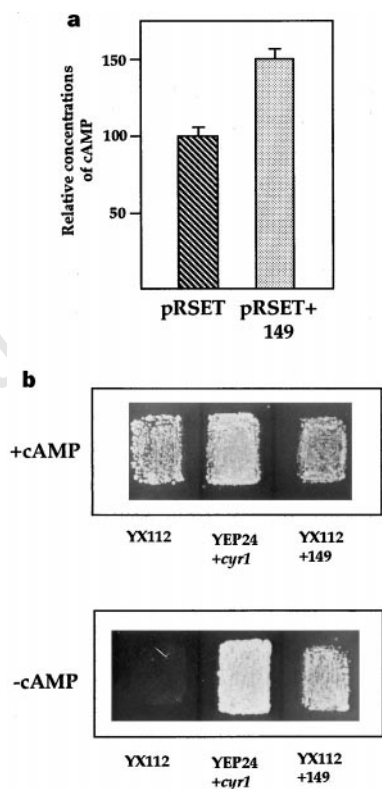


Figure 3 Functional testing of *axi 141*. **a**, Expression in *E. coli*. cAMP in extracts of bacteria transformed with pRSET containing no insert (pRSET), or pRSET containing the *axi 141* coding region (pRSET + 149). Error bars indicate standard deviation of six independent *E. coli* transformants. Difference significant at $P = 0.01$. **b**, Functional complementation of the yeast CRY1 mutant. Yeast cultured at 35°C in the absence or presence of cAMP. Left to right, T50-3A transformed with pYX112, T50-3A transformed with pYEP24 + *cyr1*, and T50-3A transformed with pYX112 + 149. Top, with cAMP; bottom, without cAMP.

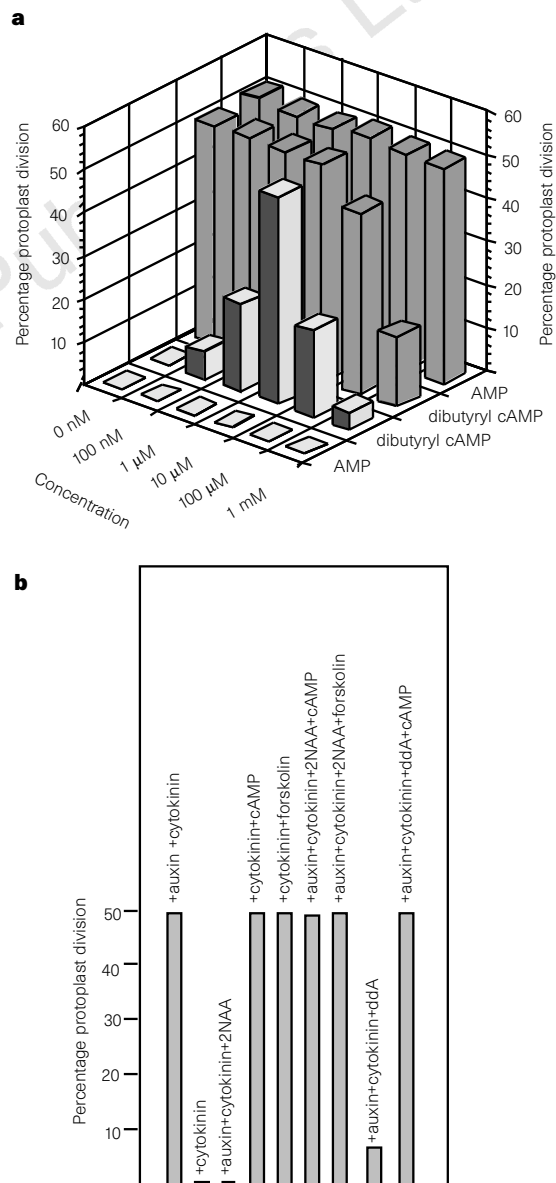


Figure 4 Effect of cAMP on protoplast division. **a**, Leaf protoplasts from untransformed plants (hatched columns) and from *axi 141* (stippled columns) and cultured in the presence of cytokinin (0.9 μ M kinetin) in increasing amounts of butyl-AMP and AMP. Protoplast division was scored microscopically after 5 days. **b**, Protoplasts from untransformed tobacco plants cultivated in auxin (5.5 μ M NAA), cytokinin (0.9 μ M kinetin), cAMP (10 μ M), forskolin (400 μ M) and dideoxyadenosine (100 μ M). Protoplast division was scored microscopically after 5 days.

with 57% identity to *axi 141* (data not shown), encoded in part by an ORF located on chromosome IV of *Arabidopsis* (PID: g2244920), which is probably an *axi 141* homologue. This ORF also lacks membrane-spanning regions, and scanning the sequence up to 15 kb 5' of the ORF does not uncover an ORF containing obvious membrane-spanning regions. Thus it seems that the adenylyl cyclase is soluble. An adenylyl cyclase isolated from the plant symbiont *Rhizobium* is also a soluble enzyme¹⁷.

Although little is understood of the auxin signal-transduction pathway, current models hold that auxin, detected at the plasma membrane, triggers changes in ion transport as well as gene expression¹⁸. The finding that cAMP may be involved in this is significant because, by analogy with other experimental systems, it may provide an intermediary signal capable of activating both processes. In mammals, cAMP causes changes in ion transport¹⁹ as well as in gene expression²⁰. Indeed, cAMP has been found to stimulate K⁺ channel activity in beans²¹, and several genes encoding proteins with similarities to mammalian cAMP response element-binding protein/activating transcription factors (CREB/ATF) have been found in plants²². Generally, cAMP activates protein kinase A (PKA), which in turn initiates a phosphorylation cascade¹. So far, although genes with similarities to PKA and PKC have been isolated from plants³, no plant PKA has been identified definitively. With the idea that cAMP is involved in triggering an auxin response, identification of the PKA responsible is clearly a future challenge. But our finding that a plant adenylyl cyclase is involved leads to the speculation that cAMP levels may be controlled by guanine nucleotide-binding proteins (G proteins)¹. Several of these have already been identified in plants, although their exact role is yet to be established²³. The observations that higher plants have an adenylyl cyclase and that cAMP can act in auxin signalling not only provides information about auxin action, but also provides the tools and suggests strategies to isolate other components of the signal-transduction pathway leading to auxin-induced cell division. □

Methods

Plant tissue culture. Activation tagging was carried out in tobacco (SR1) using pPCVICEN4HPT⁴. Protoplasts from leaf tissue¹² were cultured in auxin (5.5 µM NAA; Sigma) and cytokinin (0.9 µM kinetin; Sigma), or in cytokinin alone plus cAMP (Sigma), dibutyl-AMP (Sigma), forskolin (Sigma) and dideoxyadenosine (Calbiochem) at the concentrations indicated. The proportion of protoplasts undergoing division 5 days after isolation was judged microscopically using a Neubauer cell counting chamber (Fastnach) at least three times. Variation between each sample was less than 10%.

Recombinant DNA techniques. Routine cloning was carried out in *E. coli* DH5 α, and plasmid rescue was performed²⁴ using *E. coli* DH10B (Electromax, Gibco/BRL) by electroporation as recommended by the manufacturer. A λgt 11D (Pharmacia) cDNA library was constructed using poly(A)⁺ RNA from tobacco protoplasts 2, 3 and 5 days old cultivated in NAA (K. Fritze and H. Harling, unpublished). Positive clones were subcloned in pBluescript II SK (+) (Stratagene). Southern analysis was performed using genomic DNA from leaf tissue²⁴. RNA was isolated from protoplasts with RNeasy (Qiagen). RNA probes were transcribed from pB149 (digested with *Xho*I) by T3 polymerase using a Riboprobe transcription kit (Promega). Expression in protoplasts was carried out using the expression vector pRT106 (ref. 25).

Measuring cAMP levels in *E. coli*. The *Eco*RI–*Sac*I fragment of pB149, lacking 28 amino acids of the N terminus of *axi 141*, was cloned as a translational fusion with a six-histidine tag in pRSET A (Invitrogen). This was transformed into *E. coli* BL21(DE3)pLysS (Stratagene). Expression was induced by treatment with IPTG²⁶. After 90 min, cAMP was recovered using an Amprep

SAX column (Amersham) and measured by a BIOTRAK EIA kit (Amersham). Simultaneously, bacterial extracts were tested for the presence of the recombinant protein by western analysis using an anti-six-histidine-tag antibody (Qiagen).

Yeast complementation. The *Eco*RI–*Sac*I fragment of pB149 was cloned into pYX112 (Ingenium) and pYX112–149 was transformed into the *Saccharomyces cerevisiae* CYR1 ts sensitive mutant T50–3A¹⁰ at the non-permissive temperature of 35 °C. Surviving clones were tested for cosegregation of the recombinant plasmid and growth under non-permissive conditions. As a control, T50–3A was transformed with either the yeast CYR1 gene¹⁰ cloned in pYEP24 (ref. 27) or pYX112 and grown at either 35 or 25 °C.

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